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Contents of Volume 160

No. 1. JANUARY 1950

HEMOGLOBIN IRON AS A STIMULUS FOR PRODUCTION OF FERRITIN BY KIDNEY	<i>John K. Hampton, Jr. and H. S. Mayerson</i>	1
COMPARISON OF EFFECTS OF SODIUM PENTOBARBITAL OR ETHER-INDUCED ANESTHESIA ON RATE OF FLOW AND CELL CONTENT OF RAT THORACIC DUCT LYMPH	<i>Gerald F. Hungerford and William O. Reinhardt</i>	9
MECHANISM OF HYPOTENSION FOLLOWING INTRAVENOUS INJECTIONS OF STRONGLY HYPERTONIC SOLUTIONS	<i>William W. Walcott and Ingrith J. Deyrup</i>	15
BLOOD PRESSURE AND RENAL CLEARANCES IN HYPERTENSIVE DOGS FOLLOWING TISSUE INJURY	<i>J. Stamler, S. Rodbard and L. N. Katz</i>	21
IMPORTANCE OF DIETARY PROTEIN, CALORIES AND SALT IN EXPERIMENTAL RENAL HYPERTENSION	<i>Philip Handler and Frederick Bernheim</i>	31
FAILURE OF PORTAL SYSTEM TO INACTIVATE COMPLETELY OVARIAN SECRETIONS IN IMMATURE RATS	<i>N. T. Werthessen and N. S. Field</i>	41
ACTOMYOSIN OF THE UTERUS	<i>A. Csapó</i>	46
COMPARISON OF PROTEIN ANABOLIC PROPERTY OF VARIOUS ANDROGENS IN CASTRATED RAT	<i>Charles D. Kochakian</i>	53
COMPARISON OF PROTEIN ANABOLIC PROPERTY OF TESTOSTERONE PROPIONATE IN MALE AND FEMALE RAT	<i>Charles D. Kochakian and Betty Beall</i>	62
COMPARISON OF PROTEIN ANABOLIC PROPERTIES OF TESTOSTERONE PROPIONATE AND GROWTH HORMONE IN RAT	<i>Charles D. Kochakian</i>	66
LEUCOCYTE PICTURE OF RAT: RELATION OF ADRENAL AND SPLEEN	<i>Abraham Dury</i>	75
EFFECT OF DESOXYCORTICOSTERONE ACETATE AND ADRENAL CORTICAL EXTRACTS ON SURVIVAL OF ADRENALECTOMIZED AND INTACT RATS AFTER BURNING	<i>S. S. You and E. A. Sellers</i>	83
MECHANISM OF EXTRACELLULAR SODIUM AND CHLORIDE DEPLETION IN ADRENALECTOMIZED DOG	<i>James B. Flanagan, A. K. Davis and R. R. Overman</i>	89
PRODUCTION OF DIABETES IN MOUSE BY PARTIAL PANCREATECTOMY	<i>Frances Pauls and Richard W. Bancroft</i>	103
IMPAIRMENT OF LIVER GLUCONEOGENESIS FROM LACTIC ACID AS RESULT OF ALLOXAN ACTION	<i>R. Carrasco-Formiguera and Marco Tulio Mendoza</i>	107
EFFECT OF LIGATION OF PANCREATIC DUCT UPON ACTION OF SECRETIN AND PANCREOZYMIN IN RABBITS WITH CORRELATED HISTOLOGICAL STUDY	<i>C. C. Wang, K. J. Wang and M. I. Grossman</i>	115

EFFECT OF TEMPERATURE UPON SURVIVAL OF EVISCERATE RAT	
<i>Dwight J. Ingle and James E. Nezamis</i>	122
OXYGEN TRANSPORT AND UTILIZATION IN DOGS AT LOW BODY TEMPERATURES	
<i>W. G. Bigelow, W. K. Lindsay, R. C. Harrison, R. A. Gordon</i> <i>and W. F. Greenwood</i>	125
INTERRELATIONSHIP OF CARDIO-RESPIRATORY EVENTS IN ANOXIA	
<i>M. Brucer, Gloria L. Herman and H. G. Swann</i>	138
CORONARY BLOOD FLOW AND CARDIAC OXYGEN CONSUMPTION IN UNANESTHETIZED DOGS	
<i>F. C. Spencer, D. L. Merrill, S. R. Powers and R. J. Bing</i>	149
DIRECT DETERMINATION OF PARTIAL AND TOTAL TENSIONS OF RESPIRATORY GASES IN BLOOD	
<i>Albert Roos and Harrison Black</i>	163
EFFECT OF AURICULAR FIBRILLATION ON CORONARY BLOOD FLOW	
<i>René Wégria, Richard P. Keating, Henry P. Ward</i> <i>Fritz Dreyfuss, Charles W. Frank and</i> <i>Marvin R. Blumenthal</i>	177
COMPARISON BETWEEN CARDIAC INPUT MEASURED WITH ROTAMETER AND OUTPUT DETERMINED BY DIRECT FICK METHOD IN OPEN-CHEST DOGS	
<i>R. A. Huggins, E. L. Smith and M. A. Sinclair</i>	183
INHIBITION OF RAT BRAIN CHOLINESTERASE BY β -CHLORINATED AMINES	
<i>James A. Bain</i>	187
CONVULSIONS IN CEREBELLUM AND CEREBRUM INDUCED BY β -CHLORINATED AMINES	
<i>George H. Pollock and James A. Bain</i>	195
EFFECT OF STIMULATION ON PHOSPHATE ESTERS OF BRAIN	
<i>R. M. C. Dawson and D. Richter</i>	203
SYMPATHOLYTIC EFFECTS OF QUININE AND QUINIDINE	
<i>Edwin P. Hiatt</i>	212

No. 2. FEBRUARY 1950

ANTAGONISM OF ADRENOCORTICOTROPHIC HORMONE AND ADRENAL CORTICAL EXTRACT TO DESOXYCORTICOSTERONE: ELECTROLYTES AND ELECTROSHOCK THRESHOLD	
<i>Dixon M. Woodbury, Chi-Ping Cheng, George Sayers and</i> <i>Louis S. Goodman</i>	217
INITIAL CHANGES IN BLOOD SUGAR OF FASTED ANESTHETIZED DOG AFTER ALLOXAN	
<i>G. A. Wrenshall, Jean Collins-Williams and C. H. Best</i>	228
CHANGES IN BLOOD OF RAT FOLLOWING EVISCERATION	
<i>Dwight J. Ingle, Mildred C. Prestrud and James E. Nezamis</i>	247
INFLUENCE OF VARIATION IN ENVIRONMENTAL TEMPERATURE AND THYROID STATUS ON GROWTH AND FEED CONSUMPTION OF MALE MOUSE	
<i>M. Maqsood and E. P. Reineke</i>	253
UTILIZATION OF PURE α -, γ -, AND δ -TOCOPHEROLS BY LAYING HENS	
<i>Mei Yu Dju, Mary L. Quaipe and Philip L. Harris</i>	259
EFFECTS OF SOME EXCHANGE RESINS ON MINERAL METABOLISM OF RATS	
<i>Evan W. McChesney and John P. McAuliff</i>	264

CHANGES IN BLOOD OF RAT DURING ETHER AND BARBITURATE ANESTHESIA	<i>Henry I. Kohn</i>	277
EMOTIONAL HYPOTHERMIA IN RABBITS	<i>Ronald Grant</i>	285
DO KANGAROO RATS THRIVE WHEN DRINKING SEA WATER?	<i>Bodil Schmidt-Nielsen and Knut Schmidt-Nielsen</i>	291
WATER AND ELECTROLYTE DISTRIBUTION IN BLOOD AND TISSUES IN SPLENECTOMIZED DOGS BEFORE AND AFTER HYPOTONIC SALINE INJECTIONS	<i>Lillian Eichelberger and Michael Roma</i>	295
RELATION OF GLOMERULAR FILTRATION RATE AND SODIUM TUBULAR REJECTION FRACTION TO RENAL SODIUM EXCRETION	<i>D. M. Green, W. C. Bridges, A. D. Johnson, J. H. Lehman, F. Gray and L. Field</i>	306
RENAL TUBULAR ELIMINATION OF N ¹ -METHYLNICOTINAMIDE	<i>Karl H. Beyer, Horace F. Russo, S. R. Gass, Katharine M. Wilhoyle and Alice A. Pitt</i>	311
CHANGES IN RENAL FUNCTIONS ASSOCIATED WITH DIABETES INSIPIDUS PRECIPITATED BY ANTERIOR HYPOTHALAMIC LESIONS	<i>Carroll A. Handley and Allen D. Keller</i>	321
RENAL CLEARANCE OF FERROCYANIDE IN DOG	<i>Robert W. Berliner, Thomas J. Kennedy, Jr. and James G. Hilton</i>	325
CITRIC ACID AND ITS RELATION TO SERUM AND URINARY CALCIUM	<i>T. S. Chang and Smith Freeman</i>	330
ROLE OF KIDNEY AND OF CITRIC ACID IN PRODUCTION OF A TRANSIENT HYPERCALCEMIA FOLLOWING NEPHRECTOMY	<i>Smith Freeman and T. S. Chang</i>	335
EFFECT OF THYROPARATHYROIDECTOMY AND VITAMIN D UPON SERUM CALCIUM AND CITRIC ACID OF NORMAL AND NEPHRECTOMIZED DOGS	<i>Smith Freeman and T. S. Chang</i>	341
ANTIPROTEOLYTIC ACTIVITY OF SERUM FROM DOGS WITH EXPERIMENTALLY-INDUCED PEPTIC ULCERS	<i>Eugene E. Clifton and Louis E. Young</i>	348
EFFECT OF INTRAVENOUS SODIUM SULFATE ON RENAL EXCRETION IN DOG	<i>A. V. Wolf and Stanley M. Ball</i>	353
ANOXIA IN EXPLOSIVE DECOMPRESSION INJURY	<i>W. M. Hall and E. L. Corey</i>	361
ELECTRICAL ALTERNATION IN EXPERIMENTAL CORONARY ARTERY OCCLUSION	<i>Herman K. Hellerstein and Irving M. Liebow</i>	366
TEMPORAL SUMMATION OF STIMULI STUDIED WITH THE AID OF ANTICHO-LINESTERASES	<i>Robert Gesell and Jeane Siskel Frey</i>	375
PONTINE AND MEDULLARY REGULATION OF RESPIRATION IN THE CAT	<i>C. G. Breckenridge and Hebbel E. Hoff</i>	385
PHYSIOLOGICAL DETERMINATION OF BOUNDARY OF ACOUSTIC AREA IN CEREBRAL CORTEX OF THE DOG	<i>Archie R. Tunturi</i>	395
THERMOSENSITIVITY OF TURTLE BRAIN AS MANIFESTED BY BLOOD PRESSURE CHANGES	<i>S. Rodbard, F. Samson and D. Ferguson</i>	402

PRESSOR SUBSTANCES IN ARTERIAL HYPERTENSION: ACTIVITY AND AMINE CONTENT OF CRUDE EXTRACTS OF BLOOD	<i>C. Chester Stock and Henry A. Schroeder</i>	409
INFLUENCE OF LIVER ON VASCULAR REACTIVITY	<i>Irvine H. Page</i>	421
RELATIONSHIP OF PORTAL HYPERTENSION AND IRREVERSIBILITY OF SHOCK	<i>Roy Cohn and Harry Parsons</i>	437
No. 3. MARCH 1950		
FUNCTIONAL SIGNIFICANCE OF RESIDUAL SYMPATHETIC PATHWAYS FOLLOW- ING VERIFIED LUMBAR SYMPATHECTOMY	<i>W. C. Randall, W. F. Alexander, A. B. Hertzman, J. W. Cox and W. P. Henderson</i>	441
EFFECT OF SOME DRUGS ON POLARIZATION STATE OF SPINAL CORD ELEMENTS	<i>A. Van Harreveld and George A. Feigen</i>	451
FORMATION OF CONDITIONED AVOIDANCE RESPONSES IN YOUNG PUPPIES	<i>John L. Fuller, Clarice A. Easler and Edwin M. Banks</i>	462
PILOCARPINE SENSITIZATION IN PARASYMPATHETICALLY DENERVATED PUPIL OF CAT	<i>Enid Anne Neidle</i>	467
RESPONSE OF PARASYMPATHETICALLY DENERVATED CAT'S PUPIL TO ESERINE	<i>Enid Anne Neidle</i>	474
MECHANISM OF ACUTE LETHAL EFFECT OF EPINEPHRINE IN RATS	<i>Mark Nickerson, John Berghout and Robert N. Hammerstrom</i>	479
ADRENALINE APNEA IN MEDULLARY ANIMAL	<i>Hebbel E. Hoff, C. G. Breckenridge and J. E. Cunningham</i>	485
ADRENERGIC AGENTS AND ADRENOCOTROPIC ACTIVITY OF ANTERIOR PITUI- TARY	<i>Ethel Ronzoni and Seymour Reichlin</i>	490
SODIUM PENTOBARBITAL ANESTHESIA AND RESPONSE OF ADRENAL CORTEX TO STRESS	<i>Ethel Ronzoni</i>	499
EFFECT OF WORK UPON TOLERANCE OF NORMAL RAT FOR INTRAVENOUSLY ADMINISTERED GLUCOSE	<i>Dwight J. Ingle, James E. Nezamis and Erving H. Morley</i>	506
STUDIES ON MECHANISM OF POTENTIATION OF CIRCULATORY EFFECTS OF HYPERTONIC SOLUTIONS RESULTING FROM ADMIXTURE WITH HOMOL- OGOUS BLOOD	<i>Ingrith J. Deyrup and William W. Walcott</i>	509
OBSERVATIONS ON HYPOTENSION FOLLOWING INTRAVENOUS INJECTION OF STRONGLY HYPERTONIC SOLUTIONS MIXED WITH HOMOLOGOUS BLOOD	<i>Ingrith J. Deyrup and William W. Walcott</i>	519
MEASUREMENT OF EXTRACELLULAR FLUID BY MEANS OF CONSTANT INFUSION TECHNIQUE WITHOUT COLLECTION OF URINE	<i>Irving L. Schwartz</i>	526
MOVEMENT OF INULIN BETWEEN PLASMA AND INTERSTITIAL FLUID	<i>David Schachter, Norbert Freinkel and Irving L. Schwartz</i>	532
CHLORIDE EXCRETION DURING OSMOTIC DIURESIS IN DOG	<i>Louis J. Cizek and Joseph H. Holmes</i>	536

SIMULTANEOUS COMPARISON OF RENAL BLOOD FLOW AS MEASURED BY FICK PRINCIPLE AND BY BUBBLE FLOW METER	<i>Hadley L. Conn, Jr. and Kehl Markley</i>	547
REFLEX CARDIAC INHIBITION IN GANOID ACIPENSER STURIO	<i>Bruno Kisch</i>	552
EFFECT OF NORMAL AND ABNORMAL CHANGES OF INTRATHORACIC PRESSURE ON EFFECTIVE RIGHT AND LEFT ATRIAL PRESSURES	<i>David F. Opdyke and Gerhard A. Brecher</i>	556
QUANTITATIVE STUDY OF REDUCTION OF GASTRIC ACID SECRETION ASSOCIATED WITH PYREXIA	<i>D. Blickenstaff and M. I. Grossman</i>	567
NATURE OF ACTION OF LABILE FACTOR IN FORMATION OF THROMBIN	<i>Armand J. Quick and Mario Stefanini</i>	572

3 1 4 3

7-3

5 5.

6-9

5-10

9-12



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HEMOGLOBIN IRON AS A STIMULUS FOR THE PRODUCTION OF FERRITIN BY THE KIDNEY¹

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THERE has been accumulated during the last few years considerable evidence which emphasizes the importance of ferritin in the storage of iron. Granick and his colleagues (1) have shown that ferrous iron entering the mucosal cells of the gastrointestinal tract brings about a rapid accumulation of apoferritin to which the iron attaches itself in the form of micelles of ferric hydroxide, the resulting compound being called 'ferritin.' These workers showed that iron absorbed by the liver, spleen and bone marrow is stored mainly in the form of ferritin.

The present study was designed to investigate the role of ferritin in the handling of iron by the renal tissue, particularly the handling of iron of Hb. Although a considerable amount of work has been done on the problem of hemoglobinuria and of athrocytosis by the kidney epithelium, relatively little information is available as to the form in which the iron of Hb. is held in the kidney. The intracellular splitting of the Hb. molecule has been shown to occur after its reabsorption into renal epithelial cells (2). Such splitting involves the release of histochemically demonstrable iron which reaches a maximum about 65 hours following intraperitoneal injection of a large quantity of Hb. in solution and this iron slowly regresses thereafter. Our experiments indicate that much of this iron is present in the form of ferritin.

METHODS

Hb. in solution was prepared according to a modification of the method of Hamilton *et al.* (3). Whole blood (either human or dog) was centrifuged, the plasma

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¹ MATERIAL TAKEN from a thesis presented to the Graduate School of Tulane University by John K. Hampton, Jr., in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report was given at the meetings of the American Physiological Society in Detroit in April 1949. Aided by a grant from the Medical Research Fund of Tulane University.

removed and the cells washed twice with 0.9 per cent NaCl. Distilled water was added to the packed cells in a ratio of approximately 1.5 parts water to one part packed cells. After laking, most of the stroma was removed by reducing the pH of the mixture to between 5.7 and 5.8 by the careful addition of 0.1N HCl. As the pH approached 5.8, a heavy protein coagulum became evident which aggregated, when allowed to stand for a few minutes, into a thick mass of material. This was separated by repeated filtration to insure as complete a removal of particulate material as possible. The pH of the filtrate was returned to 7.0 to 7.4 by the addition of saturated NaHCO_3 solution and sterilized by filtration under pressure through a Seitz filter. The final solution was transferred to sterile rubber-capped bottles and stored at approximately 4°C. Hb. iron was determined on each solution by the colorimetric method of Wong (4). Iron concentrations were usually between 20 to 22 mg/100 ml.

Estimation of the amount of ferritin in tissues was carried out by the semi-quantitative method first used by Granick (5). With slight modification, the method was as follows: After preparing two slides of each tissue by mincing approximately 0.1 gm. of tissue with two drops of 10 per cent cadmium sulfate and covering each with a coverslip, the slides were placed in a moist chamber overnight. Each slide was examined under 'high dry' power of the microscope. The relative amounts of ferritin crystals formed were recorded as trace, little, moderate, much, or very much, and in that order, the symbols one through five plus applied. Since the magnitude of difference from one slide to another often was so great, from the absence or rarity of crystals to many hundred, it is felt that this method was significantly accurate. The experience of examining hundreds of such slides aided the development of consistency in assigning values and crystal size could, to some extent, be taken into account. As will be seen in the data reported, the dose of Hb. can be related not only to the concentration of ferritin, but also to the percentage of animals showing detectable concentrations in the various tissues, especially the kidney.

EXPERIMENTAL OBSERVATIONS

Experiments on Mice. Male and female mice of the Wistar strain, weighing between 24 and 28 grams, were used. They were maintained on Rockland Mouse Diet containing approximately 37 mg. of iron per 100 grams. The sterile Hb. solutions were injected intraperitoneally and the animals killed at the conclusion of the experiments by an overdose of Nembutal.

Preliminary experiments indicated that the mouse kidney was capable of producing ferritin after the intraperitoneal injection of Hb. solution. Further experiments indicated that a dosage of about 0.1 mg. of Hb. iron per mouse with an average weight of 25 grams is close to the 'threshold' dose for the production of ferritin in the kidney. These experiments also indicated that 3 days was the optimal time for killing animals after the last injection. The studies of Rather (2) also indicated that histochemically demonstrable iron in the kidney reached its maximum about 65 hours after intraperitoneal injection of hemoglobin.

In order to determine the minimal dose of Hb. iron necessary to produce crystallizable ferritin in all of the animals treated, 6 mice were each injected at 3-day

TABLE 1. EXPERIMENT 4

GROUP	ANIMAL	KIDNEY		LIVER		SPLEEN	
		Females	Males	Females	Males	Females	Males
A Total dose equivalent to 0.10 mg. Fe	1	o	o	o	++++	++	+++++
	2	+	o	++	o	++++	+++
	3	o	o	o	+	o	+++
	4	+	o	+	+	+++	+++
	5	o	o	o	++++	++	+++++
	6	o	o	o	++++	+++	+++++
	7		o		+		+++
	8		o		++		+++
	9		+		++		+++++
	10		o		++		+++
B Total dose equivalent to 0.25 mg. Fe	1	o	+	o	+	++	+++
	2	++	o	o	o	+++	+++++
	3	+	+	+	++++	+++	+++
	4	+	o	o	+	++	+++
	5	+	o	o	+	+++++	+++++
	6	+	o	++++	++	+++++	+++
	7		+		+		+++
	8		o		o		+++
	9		+		+++		+++++
	10		o		o		++
C Total dose equivalent to 0.35 mg. Fe	1	o	+	o	+	+++	+++
	2	++	o	o	o	+++	o
	3	+	o	o		+++++	+++
	4	++	o	++	o	+++++	++
	5	++	+	o	++	++	+++
	6	+	+	++	++	+++++	++
	7		+		+		+++
	8		+		++		++
	9		++		++		+++
D Total dose equivalent to 0.50 mg. Fe	1	+++	++	+	+++	+++++	+++++
	2	+	+	+++	++	+++	+++
	3	+	++	o		+++	+++
	4	++	+	++	+	+++++	+++
	5	+	++	o	+++	+++	+++++
	6		+++		+++		+++++
E Total dose equivalent to 0.70 mg. Fe	1	+	++	o	+	+++++	+++++
	2	++	+++	++	++	+++++	+++++
	3	+	+	+	++	+++	+++
	4	o	+	++	++	+++++	+++
	5	+	+++	+	+++	+++++	+++++
	6		++		++		+++++
	7		++		+++		+++++
	8		++		++		+++++
	9		++		+++		+++++

Semi-quantitative analysis of the ferritin concentration in the kidneys, livers and spleens of mice. Human Hb. of lots 5 and 6 were used for female and male animals, respectively. The dose was administered in 2 equal injections with a 3-day interval between them. Mice killed 3 days after the last injection.

intervals with 4 doses of Hb. solution containing 0.1428 mg., 0.1428 mg., 0.2142 mg. and 0.2856 mg. of iron, respectively. The animals were killed 3 days after the final injection. The kidneys of 5 of the animals in this series showed the presence of ferritin in a concentration similar to that in the spleen and liver. The sixth mouse showed ferritin in the spleen and liver but not in the kidney.

Further experiments along these lines showed that the amount of ferritin found in the kidney was related to the amount of Hb. injected. With small amounts (0.10 mg. of iron), ferritin was often found in the liver but not in the kidney. The failure to find ferritin in the kidney with these small doses may reflect the inability of the method to detect trace amounts. As the amount of Hb. injected was increased (0.40 mg. of iron), ferritin appeared more often in the kidney and with large amounts (0.70 mg. of iron) there was usually a significant amount of ferritin in the kidney as well as in the liver. Table 1 illustrates a typical experiment. The ratio of the mice in each group showing crystallizable ferritin to the number of animals examined is shown in table 2.

TABLE 2. RATIO OF MICE OF EACH GROUP SHOWING CRYSTALLIZABLE FERRITIN TO NUMBER OF ANIMALS EXAMINED IN EXPERIMENT 4

GROUP	KIDNEY	LIVER	SPLEEN	GROUP	KIDNEY	LIVER	SPLEEN
<i>Females</i>				<i>Males</i>			
A	2:6	2:6	4:6	A	1:10	9:10	10:10
B	4:6	2:6	6:6	B	4:10	7:10	10:10
C	4:6	2:6	6:6	C	6:9	6:8	8:9
D	5:5	3:5	5:5	D	6:6	5:5	6:6
E	4:5	4:5	5:5	E	9:9	9:9	9:9

Analysis of this data indicates that certain animals were unable to form ferritin even when large amounts of Hb. were injected. Granick and Hahn (6) also reported one of three dogs unable to form ferritin in its liver from the inorganic iron administered. This animal could take up radioactive iron into the liver in amounts comparable to the other dogs, but seemed to lack the metabolic mechanism for ferritin formation. We are unable to explain why this apparent difficulty in forming ferritin was present in two of the mice used in this experiment. We are also at a loss to explain the lower response of the female mice to the larger doses of Hb.

It is obvious that, even with the lack of great accuracy in quantitation, some increase in concentration of ferritin in the tissues occurred. To attempt to assign more than gross relative comparisons of ferritin concentrations would be unwise, since the analytical method itself is, at best, only a relative comparison. That is, an assignment of +++++ to the ferritin concentration should not be taken to indicate a concentration five times that of a tissue evaluated as +. In fact, the concentration in the former is several hundred times the concentration in the latter. Table 2 indicates that the ferritin content of the kidney more closely followed that of the liver than that of the spleen. It was expected that the spleen would show high ferritin analysis, since this organ is generally believed responsible for handling

a proportionally large part of hemoglobin split products. On the other hand, it is also quite evident that the threshold for ferritin formation (or storage) is lower for the liver than it is for the kidney.

Experiments on Rabbits. Preliminary experiments confirmed the findings of Granick (1) that, in the normal rabbit, ferritin is found in the spleen and testes but not in the liver. After the injection of human Hb., ferritin was found in the bone marrow and in the kidney. Further experiments with various doses of Hb. gave variable results which, however, indicated that the rabbit spleen and kidney were able to form ferritin when presented with sufficient Hb. iron. The following experiment illustrates the nature of the results.

Six male New Zealand White rabbits from the same breeding stock, all adults of approximately the same age and weight were used. This was an attempt to control as many variables as possible. In addition, considerable care was given to feeding, watering and maintaining clean quarters.

These animals were each given 8 intraperitoneal injections of hemoglobin solutions at 3-day intervals and killed 3 days after the final injection. The first 2 injections were calculated to give one mg. of iron per kilogram of body weight. The remaining 6 injections consisted of 2 mg. of Hb. iron per kilogram of body weight. The first 3 injections were of human Hb., lot 4, and the last 5 were of human Hb., lot 6. The total dose was 14 mg. of Hb. iron per kilogram of body weight. This total dose was selected in an attempt to use enough iron to provide a reasonable stimulus and substrate for ferritin formation. The multiple injections were intended to allow time for saturation of organs preferred for iron storage, such as liver, spleen and bone marrow, in case the kidney is not such an organ in this species.

The livers of these animals were pooled and homogenized with water. Part of the homogenate was heated to 80°C. to remove most of the protein, and the filtrate from this and a quantity of untreated homogenate were each treated with various concentrations of cadmium sulfate in an attempt to isolate crystalline ferritin. No crystals were observed on examination of any of the preparations.

The spleen and kidneys of each animal were homogenized with two parts of distilled water to one part tissue. The homogenates were heated to 80°C. and the coagulum centrifuged down. The supernatant was removed and treated with 20 per cent cadmium sulfate solution in the proportions of 25 ml. of the solution to each 100 ml. of supernatant. This is the concentration of cadmium sulfate found satisfactory for ferritin extraction from large amounts of tissue (1). As soon as the supernatant was treated, 0.01 ml. of the material was pipetted to each of two coverslips which had been rimmed with petrolatum. Slides were applied to the coverslips and the preparations were allowed to stand 48 hours for crystals to be formed. Two to 9 crystals were found on each slide of renal tissue. Only one spleen showed evidence of crystalline ferritin.

It is concluded that the kidneys of rabbits will usually form crystallizable ferritin if sufficient iron is presented to the renal tissue. The amount of iron necessary to cause crystallizable ferritin formation in renal tissue of rabbits apparently is insufficient to stimulate the production of crystallizable ferritin in rabbit spleen. However, the lack of knowledge of factors other than iron presence for stimulating ferritin

production makes it desirable to omit any conclusions other than that rabbit kidneys are able to produce ferritin when presented with sufficient iron in the form of Hb. Further experiments on rabbits are indicated.

DISCUSSION

These experiments have established the ability of the renal tissue of the mouse and rabbit to form ferritin when presented with Hb. iron.

Since Granick (1, 7) has shown ferritin in the kidneys of dogs, cats and humans, and this study extends these findings to mice and rabbits after injection of hemoglobin, it is suggested that such a mechanism is widespread among species. The available evidence suggests that normally the renal tissue is not an organ preferred for iron storage; thus the iron presented to it is shifted to other tissues such as the liver and bone marrow. Only when the kidney is presented with large amounts of iron, in face of relative iron saturation of the preferred storage sites, does it serve as a storage organ for iron. Its ability in this capacity may be limited, however, as will be pointed out later.

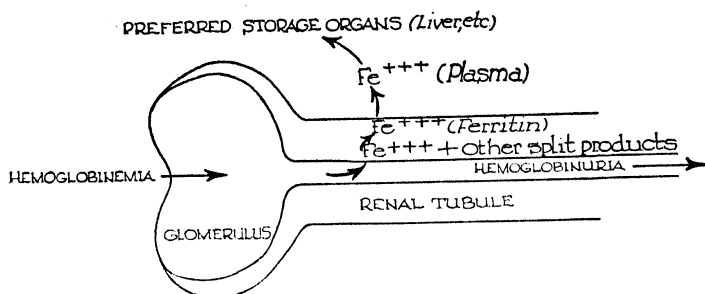


Fig. 1

The elucidation of this mechanism for handling iron in renal tissue, added to the studies of Rather on athrocytosis of Hb. by the renal tubular epithelium and its intracellular breakdown, offers convincing evidence in support of Bogniard and Whipple's (8) belief that the kidney plays an important role in conservation of Hb. iron. The kidney possesses both the ability to reclaim at least part of the hemoglobin that passes the glomerular filter and a mechanism for expeditiously handling iron when it is released from the Hb. molecule. A satisfactory study of the kinetics of such athrocytosis and elucidation of the enzymes involved in this and in the intracellular splitting which occurs would well establish our concept of the renal excretion mechanism for Hb.

Since it has been shown that ferritin functions in regulating iron absorption in the intestinal mucosa, it is suggested that in a similar manner it regulates reabsorption by the renal tubule. Just as in the gut, a reduced absorption will result until time enough has elapsed to allow recession of the ferritin iron in the renal tissue by transfer of the iron to other storage sites, so the 'lowered renal threshold' to Hb. following repeated injections may be explained as a physiological saturation of the ferritin forming mechanism. Figure 1 illustrates this concept.

It is hardly possible, with this information at hand, to avoid suggesting another factor in the pathogenesis of hemoglobinuric nephrosis. Certainly, no one factor is responsible for the various pathological findings in this condition. Prominent among the features is necrosis of the tubular epithelium. None of the previously proposed theories afford any definite answer to the etiology of this cellular destruction.

On the basis of the findings outlined above, it may be suggested that the necrosis is the result of protein precipitation by ferric hydroxide. In other words, although the kidney can take up and temporarily store iron from Hb., if, in extensive hemoglobinemia, the preferred iron storage organs become relatively saturated with stored iron, the transportation of kidney ferritin out of the kidney is slowed. When the glomerular filtrate increases in viscosity to the point of slowing down this Hb.-laden fluid, or virtual cessation of flow occurs due to cast formation, severe dehydration, decreased renal blood flow, etc., iron released from Hb. intracellularly and probably in the tubular lumen exceeds the ferritin iron handling mechanism. Such iron would tend to form ferric hydroxide in the environment of the intracellular pH.

Such a theory as presented above seems more desirable than explaining the renal damage with such vague terms as 'toxic products.' It is compatible with the newer concept of renal function, i.e. glomerular filtration followed by tubular reabsorption. Here, however, an identifiable histotoxic substance, ferric hydroxide, may become evident when the mechanism which normally protects against it is called upon to perform a greater service than it is capable of rendering. It is intended that this theory complement and supplement prevailing concepts of the pathogenesis of hemoglobinuric nephrosis, rather than replace them. No doubt several factors are involved.

The implications of our results in connection with those of Shorr and his colleagues (9, 10) are manifold. These workers have described a vasodepressor material (VDM) produced by anaerobic metabolism of various damaged and anoxic tissues, principally skeletal muscle and liver. They have recently identified this material as ferritin (11). That anoxia of a tissue causes production of VDM (or ferritin) while the same substance is formed when the stimulus is the presence of iron, finds no analogy in any other biological system. It may be that anoxia causes the release of VDM (or ferritin) rather than its production. Experiments designed to relate the various functions of ferritin are now in progress.

SUMMARY

Ferritin has been isolated from the kidney as well as from the spleen of the mouse and the rabbit after the intraperitoneal injection of Hb. solutions. Ferritin was also found in the liver of the mouse but not of the rabbit. The amount of ferritin formed was proportional to the dosage of Hb. iron used. Crystallizable amounts of ferritin were also found in the red bone marrow of the rabbit after the intraperitoneal injection of Hb. solutions.

It is suggested that when large amounts of Hb. iron are available, iron is present in the kidney in the form of ferritin. At first, the iron is split from the ferritin and is removed from the kidney and deposited in other tissues, such as liver and bone marrow. When these preferred organs for iron storage become saturated, the removal

of iron from the kidney is slowed. This may be the basis of the 'lowered excretion threshold' to Hb. noted after repeated Hb. injections. When the capacity of the ferritin iron-handling mechanism of the kidney is exceeded, some ferric hydroxide, a protein precipitant, will likely form at the intracellular pH . This may be a valid explanation of the tubular necrosis observed in renal damage from severe hemoglobinuria.

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COMPARISON OF EFFECTS OF SODIUM PENTOBARBITAL OR ETHER-INDUCED ANESTHESIA ON RATE OF FLOW AND CELL CONTENT OF RAT THORACIC DUCT LYMPH¹

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IN THE course of experiments designed to study the effects of various endocrine substances on the rate of flow and cell content of rat thoracic duct lymph, it was desirable to compare the possible effects of various anesthetic agents, and methods of lymph collection, on rats of different age groups, to determine the most satisfactory conditions for carrying out such studies.

Previous experimental work of such a comparative nature has been carried out on peripheral lymph flow in dogs. Polderman, McCarrell and Beecher (1) demonstrated that cervical lymph flow was reduced 50 per cent in quantity when collected under sodium pentobarbital anesthesia, whereas the flow was increased approximately 50 per cent under ether anesthesia, when these two states were compared with the lymph flow obtained under local procaine anesthesia. Cope and Moore (2) also observed a diminution of lymph flow in dogs after barbiturate as compared with local anesthesia. Beecher, Warren and Murphy (3) further demonstrated that cervical lymph flow in dogs under cyclopropane anesthesia was reduced as compared with the flow under ether anesthesia.

The present report presents data on rate of flow and cell content of thoracic duct lymph as influenced by two anesthetic agents, ether and sodium pentobarbital, and in the post-anesthetic state, by the use of two operative approaches for the collection of lymph, in male rats of 40, 60, and 100 days of age.

MATERIAL AND METHODS

Male rats of the Long-Evans strain were maintained on the regular laboratory diet XIV supplemented with fresh lettuce twice weekly, until lymph collections were made at the ages of 40, 60, or 100 days, in the various groups of experiments.

Two techniques were employed for the collection of thoracic duct lymph. One method (4) employed an approach to the jugular lymph sac in the neck. A glass cannula was inserted into the sac, and lymph collection was continued only while the animal was in the anesthetized state. The second method described by Bollman, Cain and Grindlay (5), involved the insertion of a plastic cannula into the thoracic duct of the anesthetized animal at a point immediately caudal to the left crus of the diaphragm, at which point the cannula was tied into place, and was drawn out through the abdominal wall. The animal was then placed in a small cage (6), allowed to recover from the anesthetic, and maintained in the cage for the duration of the

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experiment. This abdominal lymph fistula allowed collection for periods up to 10 days.

For purposes of anesthetization, a 2 per cent aqueous solution of sodium pentobarbital was administered intraperitoneally in the amount of 7 mg/100 gm. body weight. Ether anesthesia was induced in a closed chamber, and was maintained by means of the automatic apparatus described by Sawyer (7). Measurements of lymph flow were made immediately after cannulation except in those experiments in which the animals were allowed to recover from the ether anesthetized state in the case of abdominal lymph fistula collections. All cannulae were heparinized prior to use. Lymph collections were carried out for a period of 90 minutes. The quantity of lymph was measured in a graduated 1-cc. tuberculin syringe, and after mixing, samples were withdrawn for cell counts. No differential figures are presented for types of cells present in the lymph, but rather all are considered to be mononuclear white cells. All values are expressed in absolute amounts per 24 hours and in relative amounts per 100 gm. body weight per 24 hours, in order to correct for variations in weights of the various groups.

Fisher's method for determining the significance of differences of the means was employed. A difference was considered highly significant if the P value was $< .01$ and possibly significant if $< .05$.

RESULTS

Data presented in table 1 indicate the various groups of animals employed, and the absolute and relative values for lymph flow and cell count.

Comparison of Effect of Anesthetic and Post-Anesthetic State on Lymph Flow and Cell Content at Different Ages

Forty days of age. The lymph flow in the ether-anesthetized groups is both absolutely and relatively higher ($P < .01$) than the flow in the sodium pentobarbital-anesthetized rats. On the other hand, the total cell content is both absolutely and relatively decreased by the ether anesthetic in the same animals ($P < .01$). The lymph flow of the group recovering from ether anesthesia is midway between these 2 groups and is not significantly different from either. The total cell content of the post-anesthetic group, however, is higher ($P < .01$), both absolutely and relatively than either of the other 2 groups.

Sixty days of age. Lymph flow and total cell count did not differ either relatively or absolutely in any of the groups studied at this age level in the anesthetized state.

One hundred days of age. Since there was a definite difference in the effects of the two anesthetics in the 40- and 60-day-old rats, this group was studied to ascertain any possible further age difference in the effect of sodium pentobarbital on lymph flow and cell content. The results obtained do not demonstrate a significant difference in the absolute values for lymph flow and cell content as compared with the 60-day-old animals under sodium pentobarbital anesthesia. There is, however, a relative difference, based on the greater weight of the 100-day animals.

Comparison of a Single Anesthetic Agent on Lymph Flow and Cell Count at Successive Ages

Sodium Pentobarbital. There is an absolute increase in lymph flow and cell count between the ages of 40 and 60 days which levels off so that there is no significant difference between the ages of 60 and 100 days of age. Relative to body weight, however, flow is highest at 40 days of age, a gradual decline setting in up to the age of 100 days. The cell count, when related to body weight, exhibits an increase from

TABLE 1. INFLUENCE OF TYPE OF ANESTHETIC, AND METHOD OF LYMPH COLLECTION ON RATE OF FLOW AND TOTAL CELL CONTENT OF THORACIC DUCT LYMPH IN NORMAL MALE RATS

ANESTHETIC AGENT	AGE	NO. OF ANIMALS	BODY WT.	WHITE BLOOD CELLS	FLOW	TOTAL CELLS	FLOW	TOTAL CELLS
	days		gm.	per cu. mm.	cc/24 hr.	mil-lions/24 hr.	cc/100 gm/24 hr.	mil-lions/100 gm/24 hr.
Sodium pentobarbital	40	17	144 ¹ (125-180)	20,450 ¹ (3,000-40,000)	13.6 ² ±1.0	266 ² ±31	9.4 ² ±0.6	184 ² ±21
	60	14	265 (226-290)	32,750 (16,450-58,500)	22.6 ±0.9	725 ±62	8.6 ±0.4	270 ±21
	100	9	335 (280-425)	34,200 (15,900-57,000)	21.5 ±1.5	693 ±63	6.5 ±0.4	209 ±20
Ether	40	10	152 (140-165)	7,750 (2,750-17,650)	18.3 ±1.2	153 ±29	12.0 ±0.7	100 ±18
	60	8	238 (195-270)	28,900 (15,300-45,700)	22.0 ±3.3	600 ±78	9.1 ±0.9	250 ±29
Post-anesthetic (ether)	40 ³	9	155 (135-170)	32,500 (13,200-61,100)	15.8 ±1.9	495 ±70	10.1 ±1.2	322 ±47
	60 ³	9	274 (210-310)	32,150 (15,450-55,900)	25.4 ±0.8	796 ±91	9.3 ±0.3	291 ±33

¹ Mean values with range in parentheses. ² Mean values ± S. E. or mean. ³ Lymph collected by abdominal approach to the thoracic duct; all others were collected using the neck approach.

40 to 60 days of age, after which there is a decline to the 100-day level. The cell counts noted at 40 and 100 days of age are relatively lower than the peak reached at 60 days of age.

Ether anesthesia. The lymph flow between 40 and 60 days of age is relatively, but not absolutely, higher in the 40-day old groups, whereas the cell count in the 60-day old groups is significantly higher both relatively and absolutely than in the 40-day old group.

Post-anesthetic state. During the period of recovery from ether anesthesia, the lymph flow and cell count are significantly higher in the 60-day old group on an absolute, but not on a relative basis, when compared with the 40-day old group.

Comparison of Lymph Flow and Cell Count of Thoracic Duct Lymph Collected from Jugular Lymph Sac in the Neck or from the Duct in the Abdominal Cavity

A comparison of these 2 techniques for collection of lymph was made to ascertain whether there might be significant differences in the quantity and cell content of lymph collected from the thoracic duct at these two levels (table 2). In the comparable 60-day-old animals under sodium pentobarbital anesthesia, no significant difference was noted either in absolute or relative values.

DISCUSSION

The present report presents data on lymph flow and cell content of thoracic duct lymph which indicate that variations may occur in the response of the animal to the administration of either ether or sodium pentobarbital anesthetics depending on the age of the animal employed. Lymph flow in the etherized 40-day-old rat is approx-

TABLE 2. RATE OF FLOW AND CELL CONTENT OF THORACIC DUCT LYMPH OF 60-DAY-OLD MALE RATS¹

SITE OF COLLECTION	NO. OF ANIMALS	BODY WT.	WHITE BLOOD CELLS	FLOW	TOTAL CELLS	FLOW	TOTAL CELLS
				cc/24 hr.	millions/24 hr.	cc/100 gm/24 hr.	millions/100 gm/24 hr.
Neck	14	265 ² (225-290)	32,750 ² (16,450-58,500)	22.6± (0.9) ²	725± (62) ²	8.6± (0.4) ²	270± (21) ²
Abdomen	10	274 (235-310)	37,250 (21,650-52,800)	20.9± (1.1) ²	771± (58) ²	7.6± (0.3) ²	281± (21) ²

¹ Collected from the neck or abdomen of rats maintained under sodium pentobarbital anesthesia.

² Mean values with range in parentheses.

² S. E. of mean.

imately 30 per cent higher than is the flow in the animal anesthetized with sodium pentobarbital. This effect is not apparent in the 60-day-old groups. Again, the cell content of the thoracic duct of the 40-day-old rat is lower in the etherized group than is the case in the animals receiving sodium pentobarbital. The lymph flow of rats recovering from anesthetization with ether returns to a level approximating that of the sodium pentobarbital group, whereas there is a great increase in the number of mononuclear white cells in the lymph of the 40-day-old group as compared with the level in either the ether or sodium pentobarbital anesthetized animals. These changes are not apparent in the 60-day-old animals anesthetized with ether or sodium pentobarbital. Sixty-day-old animals recovering from ether anesthesia do, however, show a possibly significant higher lymph flow than do the anesthetized animals, but the absolute cell content of the lymph is not altered. These results confirm for the rat of the 40-day group, the findings previously noted in the dog (1-3).

Explanation of the changes in lymph flow under the various types of anesthetics must depend for the most part on the presumed concurrent changes in the plasma volume produced by the anesthetic agent administered. A series of reports (1, 8-10) has presented evidence to indicate that etherization is followed by a degree of hemoconcentration with presumed diversion of plasma fluid into the tissue spaces, whereas

barbiturate anesthetization may be followed by the opposite phenomenon, that is, hemodilution with diversion of tissue fluids into the plasma. Termination of the use of the anesthetic agent may then abruptly reverse the trend of flow of tissue fluid and thereby influence the rate of flow of lymph.

The differences in cell content are not explicable on the basis of a proportionate change in the rate of lymph flow. Factors to be considered as possible explanations of the differences in total cell content are among the following: 1) changes in lymph flow from various areas or organ systems of the body, as, for example, direct alteration of cell release or lymph flow from the gastroenteric tract; 2) effects of muscular activity as produced by struggling on recovery from an anesthetic as noted by Rous (11); and 3) possible participation of the hypophyseal-adrenal cortical relationship in the alarm state produced by anesthetization.

Emphasis must be placed on the variations which are encountered on the basis of the ages of the animals. It is apparent that the response of the 40-day-old rat to 2 types of anesthetic agents or recovery from ether anesthetic is far more labile than is the case with the 60-day-old rat. Exact determinations for comparative purposes of the action of various substances which may alter the rate of lymph flow or cell content of the thoracic duct lymph in the rat should be carried out on animals of a single age, with careful attention to standardization of nutritional and environmental factors. The best method of collection of lymph in short-term experiments is probably the neck preparation employing the glass cannula. The fistula is undesirable in that the recently anesthetized rat is apt to struggle and thus force out considerable numbers of cells. The abdominal lymph fistula, however, has a great advantage over the neck preparation when chemical composition of lymph and absorption studies are being made in a long-term experiment. The most convenient anesthetic for the neck preparation in our experience is sodium pentobarbital. Data presented here indicate that this anesthetic does not significantly alter the values obtained for total cells or lymph flow in the 60-day-old rat when compared with ether anesthesia or the post-anesthetic fistula preparation. Studies on the delivery of lymphocytes to the blood stream via the thoracic duct might, therefore, preferably be carried out on rats anesthetized with sodium pentobarbital employing the cervical approach to the thoracic duct.

SUMMARY

A study has been made of the rate of flow and cell content of the thoracic duct lymph of 88 normal male rats of the Long-Evans strain at the ages of 40, 60, and 100 days, under the influence of ether and sodium pentobarbital anesthetics and after recovery from the ether anesthetized state, by the use of 2 operative techniques for the collection of lymph.

Lymph flow and cell content in the 40-day-old rats are labile in response to the various anesthetic agents in comparison with the 60-day-old group in which rates of flow and cell content are not significantly altered. Thus ether anesthetization in the 40-day-old rat produces a 30 per cent absolute increase in lymph flow and a 40 per cent absolute decrease in cell content as compared with the flow and cell content under sodium pentobarbital anesthesia. These differences are not noted in the anesthetized 60-day-old groups.

Lymph collected from the thoracic duct in the neck or in the upper abdomen does not differ significantly, either for rate of flow or in cell content in 60-day-old rats under sodium pentobarbital anesthesia. Emphasis is placed on the necessity for employing animals of a single age group, sex, and comparable nutritional and environmental status in assessing the influence of any substance which may be presumed to alter either rate of lymph flow or cell content of the thoracic duct lymph.

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MECHANISM OF HYPOTENSION FOLLOWING INTRA- VENOUS INJECTIONS OF STRONGLY HYPERTONIC SOLUTIONS^{1,2}

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THE cardiovascular effects of the intravenous injection of small volumes of strongly concentrated solutions in mammals have been investigated by a number of workers in recent years. The disturbances in the circulation following such injections are varied. These include transient hypotension which may or may not occur independently of changes in heart rate (1-5). Divergence of opinion exists as to the mechanism of the characteristic brief but profound hypotension observed in anesthetized dogs following injections of concentrated solutions including hypertonic NaCl, glucose and albumin. Thus this hypotension has been attributed by some authors to vasodilatation (1, 2), and by others to weakening of the myocardium resulting from direct action of the hypertonic agents (3). The experiments described below were undertaken in an attempt to establish more definitely the mechanism participating in the development of this characteristic hypotensive response.

METHODS

Experiments were carried out on 15 mongrel dogs ranging in weight from 6.7 to 16.3 kg., and anesthetized with sodium pentobarbital (36 mg/kg. intravenously). In addition, 2 chronically sympathectomized dogs anesthetized with sodium pentobarbital were studied.³ Mean arterial pressure measurements from one of the femoral arteries were made using a standard recording mercury manometer. A Hamilton metallic membrane manometer with optical recording was used in some experiments for measurement of the systemic systolic, diastolic and pulse pressures from the opposite femoral artery. In other experiments, Hamilton manometer tracings were obtained of central venous pressure, pulmonary arterial pressure, or pulmonary venous pressure using short wide catheters or needles inserted directly into the superior vena cava, pulmonary artery, or one of the pulmonary veins which were exposed by opening the chest during intermittent positive pressure ventilation of the lungs. Sensitive rubber manometer membranes were used for recording the systemic central

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¹ Some of the data discussed in this paper were presented at the meeting of the American Physiological Society in Minneapolis, September 1948 (6).

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³ Results on cardiac rate changes in these 2 animals have been published previously (4). The sympathectomies on these dogs were performed by Drs. S. C. Wang and H. L. Borison, and we express our gratitude to them for making the animals available to us.

and pulmonary venous pressure curves. In addition, a series of 4 experiments was carried out in which the volume of one of the hind feet was determined by means of a plethysmograph consisting of a glass chamber sealed about the foot with plaster of Paris and attached through a side arm to a rubber membrane arranged for optical recording. In all experiments the injection site was the left external jugular vein, and injections of 3 to 25 ml. of hypertonic NaCl (5-20%) were made as rapidly as possible (about 3-5 ml/sec.). Control injections of 0.9 per cent NaCl were made as well. The solutions were at room temperature. It may be noted at this point that, since no significant cardiovascular effects occurred following the control injections, they will not be discussed in the results presented below. In general, other details of the experimental procedure were similar to the techniques described previously (4).

RESULTS

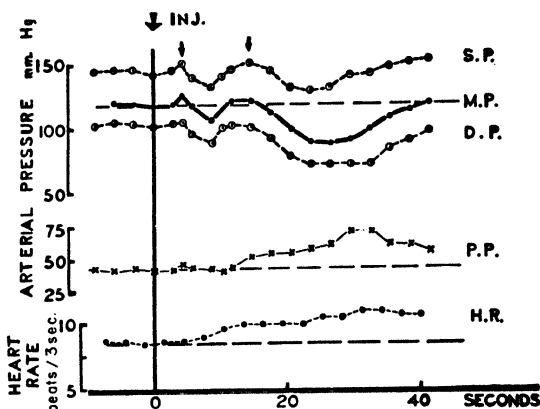
The hypotension following injection of small volumes of strongly hypertonic solutions set in within about 5 seconds. It was usually diphasic in character, with an initial moderate fall in pressure separated from a later more marked decrease by a brief period of maintenance or actual rise of pressure. Heart rates were not changed consistently. Sometimes cardiac irregularities were noted, and in most of the experiments tachycardia occurred during the period of most marked hypotension and the recovery from it. The results of a typical experiment are shown in figure 1, which summarizes the changes in femoral arterial systolic, mean, diastolic and pulse pressures, and in heart rate after intravenous injection of 3 ml. of 20 per cent NaCl into a 6.7 kg. dog. It will be observed in this figure that both systolic and diastolic pressures fell with the mean pressure, but that the pulse pressure began to rise just before the initiation of the second phase of the hypotension, and reached a peak about 28 mm. Hg above its control level of 42 mm. Hg. With the simultaneously occurring tachycardia, this increase in femoral arterial pulse pressure must be taken as evidence of systemic vasodilatation with reduction in peripheral resistance. Similar findings were obtained in a series of 5 additional experiments, and some of the results of these, as well as of the experiment shown in figure 1, are summarized in table 1. In general, a diphasic fall in mean arterial pressure occurred, and either immediately preceding, or shortly after the beginning of the secondary phase an increase in pulse pressure was noted. Before this characteristic elevation in pulse pressure set in, however, and during the primary phase of arterial hypotension, the pulse pressure varied somewhat irregularly and occasionally was decreased. As the heart rate also was often quite irregular in this early phase, it was not possible to conclude in such cases whether alteration in peripheral resistance had accompanied the first phase of the fall in blood pressure.

Observations which were consistent with the findings described above were made in 4 experiments in which the volume of one of the hind feet was measured plethysmographically (fig. 2). Following a latent period (av. 11 sec.) which was, in general, somewhat longer than the latency of the primary phase of the arterial hypotension, the limb volume decreased and then began to increase about the time when the arterial pressure had reached the minimum of the secondary depressor phase. The limb volume then increased to a value appreciably above the control level, and sub-

sequently declined towards its initial value. The conclusion was drawn from these experiments, taken in conjunction with the results described previously, that vasodilatation had occurred at least in the foot and in other portions of the vascular bed which react similarly to the vessels of the foot. It is to be noted, however, that the evidence for this vasodilatation was restricted to the period of the secondary hypotensive phase. It appeared that volume change of the foot in the earlier part of the hypotensive response might be merely passive, and parallel to the fall in mean arterial pressure.

The experiments described above gave evidence, therefore, on the mechanism of the hypotension during the later depressor phase, but no conclusion could be drawn as to the nature of the slighter primary fall in blood pressure. In the absence of any evidence for the occurrence of decreased peripheral resistance in this early phase, the possibility that the hypotension resulted from reduction in cardiac output was

Fig. 1. (Dog 12, 6.7 kg.)
EFFECT UPON FEMORAL SYSTOLIC PRESSURE (S.P.), mean pressure (M.P.), diastolic pressure (D.P.), pulse pressure (P.P.) and heart rate (H.R.) of injection into the external jugular vein of 3 ml. of 20% NaCl. The small arrows immediately above the systolic pressure curve indicate the beginning of the primary and secondary phases of the arterial hypotension.



considered. One finding which might point towards such a mechanism was the brief latency of the first phase of the hypotensive response. It seemed questionable whether the hypertonic agent could have traversed the right heart, pulmonary circulatory bed, left heart and have reached the main systemic arteries within the 3 to 6 seconds latency of the initial fall in arterial pressure. The possibility, suggested by Gennari and Levi (7), that this rapid initial phase of the hypotension might be caused reflexly following stimulation of sensory receptors in the circulatory bed in or near the heart, has been rendered highly improbable by experiments of other workers on pithed preparations (1, 3). In confirmation of the findings of the latter investigators, we have noted marked and characteristically diphasic hypotensive responses in 2 chronically sympathectomized, acutely vagotomized animals following injection of 20 per cent NaCl (table 1). If the early phase of the hypotension results from reduction in cardiac output, one or more of three possible mechanisms to account for this decrease may be considered: 1) reduction in systemic venous return; 2) reduction in pulmonary venous return, possibly initiated by pulmonary arterial spasm such as has been shown to follow histamine injection in rabbits; 3) myocardial weakening as a

result of the direct action of the hypertonic agent, or indirectly through alteration in the coronary arterial supply to the heart muscle. The second of the hypothetical mechanisms listed above seemed worthy of careful consideration since it is well known that the injection of hypertonic solutions into systemic arteries, rather than into veins, results in a marked rise in blood pressure attributed to arterial constriction. The last of the possibilities was advocated by Muirhead *et al.* (3) as the sole cause of hypertonic solution hypotension.

In an attempt to analyze the part played by these hypothetical cardiovascular alterations in the observed arterial depressor response, measurements of systemic central venous pressures, pulmonary venous pressures, and pulmonary arterial pressures were made in 5 experiments. No striking characteristic changes in any of these pressures were observed at any time during the period of reduction in systemic arterial pressure. Pulmonary arterial systolic and diastolic pressures were unchanged or raised very slightly, and pulmonary pulse pressure, if it changed at all, showed merely a slight elevation. Although barely measurable increases in systemic and

TABLE 1. CHARACTERISTICS OF HYPOTENSIVE RESPONSE TO INJECTION OF 20 PER CENT NaCl

	VOL. OF 20% NaCl INJEC- TED	FIRST PHASE		SECOND PHASE	
		Latency	Maximum Fall in Pressure	Latency	Maximum Fall in Pressure
	ml.	sec.	mm. Hg	sec.	mm. Hg
Normal dogs (6) (6.7-11.2 kg.)	3-4	3.0-6.0 (4.5)	12-22 (16)	8.3-18.3 (14.0)	18-44 (32)
Chronically sympathectomized, acutely vagotomized dogs (2) (10-11 kg.)	12	6.0-11.0	78-108	30.0-31.2	72-100

Numbers in parentheses represent averages.

pulmonary venous pressures were noted to occur approximately simultaneously with the fall in systemic arterial pressure, the small magnitude of the changes rendered definite interpretation of them difficult. On the other hand, the fact that no striking elevation in systemic central venous or pulmonary arterial pressures occurred appears to rule out the possibility of an acute decrease in venous return to the left side of the heart by pulmonary arterial spasm. Probably this is the only definite conclusion which may be drawn as to the mechanism of the decreased cardiac output in the early phase of the arterial hypotension following hypertonic NaCl injections.

In analyzing the results of the measurements of pressure in the great vessels within the thorax, objection may be raised on the basis that the open chest preparation is, unquestionably, not a normal physiological system. Whereas this is fully recognized, it is to be noted that the pattern of the hypotensive response in these open chest experiments did not differ significantly from the fall in blood pressure seen in normal anesthetized animals. It was inferred, therefore, that the mechanisms causing the hypotension in normal animals must be operating in the case of the preparations with open chest as well. In the absence of more direct evidence, and in view of the evidence from other lines of attack upon the problem presented by Muirhead *et al.* (3),

myocardial weakening may be considered as a probable mechanism for the early fall in pressure. This myocardial weakening, if it occurs, apparently is not of sufficient magnitude to cause striking elevation in either systemic central or pulmonary venous pressures.

DISCUSSION

The controversy as to the mechanism of action of hypotonic solutions upon the arterial pressure may be resolved into two main points of view. Bernstein (1) believed that the effect was one of vasodilatation as evidenced by increased pulse pressure, an action which was independent of the central nervous system since it was demonstrable in the pithed dog. Muirhead and his co-workers (3), on the other hand, attributed the action to a depressant effect on the heart. They found, among other things, that injections did not produce hypotension unless they were introduced into the venous side of the circulation, the heart, the root of the aorta or into the coronary arteries so that the solutions reached the heart without traversing a sys-

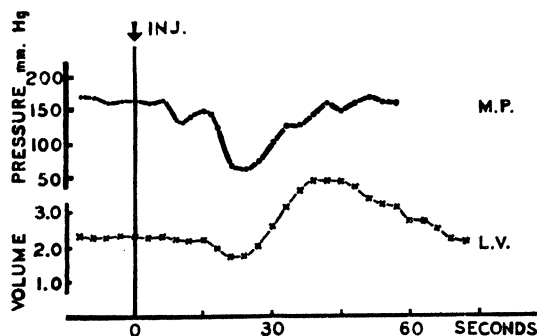


Fig. 2. (Dog 27, 16.1 kg.) EFFECT UPON FEMORAL MEAN ARTERIAL PRESSURE (M.P.) and the volume of the hind foot (L.V.) of the intravenous injection of 10 ml. 20% NaCl. The units of volume change (plethysmographic curve) are arbitrary, representing linear distance in cm. on the optical recording.

temic capillary bed. They noted, moreover, that plethysmographic records of volume changes of intestinal loops, the spleen and kidney gave no evidence of vasodilatation. The results outlined in the present communication are in agreement, in part, with both sides of this controversy. It appears from our data that vasodilatation does occur and can be demonstrated both by means of the increased systemic pulse pressure and in the increased volume of the hind foot following injections of 20 per cent NaCl. The fact that Muirhead *et al.* were unable to demonstrate it may be attributed, perhaps, to their choice of visceral organs only as objects for their plethysmographic studies. It appears possible that intra-abdominal blood vessels may not respond to the hypertonic agent in the same manner as do other systemic vessels. At the same time, vasodilatation is not demonstrable early in the hypotensive response. The brevity of the latency of the hypotension, and our failure to establish any other mechanism accounting for the early fall in systemic pressure with unchanged or reduced pulse pressure lends support to the hypothesis that reduction in cardiac output plays a role, at least in the early phase of the hypotension. The overall mechanism appears to be quite complicated, therefore, and to involve a variety of cardiovascular alterations whose interaction results in the final characteristic diphasic pattern of arterial hypotension.

SUMMARY

An analysis of the mechanism of the rapid but brief hypotension following the intravenous injection of hypertonic NaCl in anesthetized dogs suggests that at least two factors enter into the phenomenon. The first of these appears to be decreased cardiac output which may result from weakening of the myocardium, although our experiments have not enabled us to draw definite conclusions on this point. The second mechanism is reduction in peripheral resistance, which appears to play a part in the later and more marked phase of the arterial hypotension.

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BLOOD PRESSURE AND RENAL CLEARANCES IN HYPERTENSIVE DOGS FOLLOWING TISSUE INJURY¹

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SINCE the production of experimental renal hypertension by Goldblatt (1), numerous attempts have been made to lower the blood pressure to normal levels with renal extracts (2, 3). In critically evaluating the occasional positive results obtained, it has been pointed out (2, 3) that the blood pressure reductions may be the result of a non-specific 'pyrogenic' reaction, rather than a response to specific depressor agents in the renal extracts. Previous work in this laboratory on the depressor effects of subcutaneous tissue implantations and of distemper (4-7) led to similar conclusions.

Beyond these observations, little work has been done to elucidate the mechanism of these non-specific depressor reactions. Taylor and Page (8) demonstrated that neither leucocytosis nor fever are the hypotensive etiologic agents. Others have studied changes in kidney function following administration of renal extracts (9) or pyrogens (10, 11). Despite these studies, the depressor mechanism remains to be elucidated.

In pursuing the problem further we have studied *a*) the effect of several types of tissue damage on blood pressure in spontaneous and nephrogenic hypertensive dogs and *b*) the changes in renal clearances accompanying the blood pressure alterations.

METHODS

Fifty-seven experiments on the effects of various types of tissue injury on the blood pressure were carried out on 35 hypertensive mongrel dogs (table 1). Ten of these animals were spontaneous hypertensives (4, 12) and 25 were Goldblatt animals. Blood pressures were recorded on these trained, unanesthetized animals with the Hamilton manometer according to the technique previously described (4). The blood pressure was determined weekly in most cases on all dogs and 3 to 5 times weekly during the course of an experiment. Dogs were designated as spontaneous hypertensives when a blood pressure in excess of 185/100 mm. Hg with a slow pulse was maintained after weeks of training (4, 12). Nephrogenic Goldblatt hypertension was produced by partial constriction of both renal arteries with linen ligatures (1, 4-7, 12). A slightly positive depressor response following tissue injury was one in which both systolic and diastolic blood pressures fell at least 20 mm. Hg and remained depressed below control level for at least 3 successive days; a definitely positive response was one in which the drop in pressure continued for 5 days or longer, with the body temperature being normal during at least part of this time.

In 12 experiments on 8 dogs repeated determinations of renal plasma flow and glomerular filtration rate were accomplished prior to and at intervals following tissue injury (table 2). Four of these

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dogs (Z14, Z36, Z93, Z95) were nephrogenic hypertensives and 4 (Z11, Z15, Z20, Z39⁴) were spontaneous hypertensives. Repeat experiments on the same dog were done at intervals of 2 to 10 months. All renal clearances were done in the unanesthetized postabsorptive animal, using sodium para-aminohippurate (PAH) for the determination of renal plasma flow (RPF) and creatinine for the estimation of glomerular filtration rate (GFR). The renal clearance methods have been fully described in the previous paper (12).

In several of these animals, food and water intake, body weight and rectal temperature were recorded daily for a period prior to and following production of tissue injury. Hemoglobin (Sahli), erythrocyte sedimentation rate (e.s.r.) and hematocrit (Wintrobe), total white blood cell count and differential were also recorded serially in some dogs.

RESULTS

The data on the effect of various tissue-damaging agents on the blood pressure of hypertensive dogs are summarized in table 1.

Bacterial Agents. It is evident that the various intravenously administered bacterial agents including typhoid, streptococcus and staphylococcus vaccines had no sustained depressor effect in the dosage used despite the fact that sufficient material was administered to produce an acute pyrogenic reaction in some dogs and to render them clinically ill for 24 to 72 hours. At most, these animals exhibited an ephemeral fall in blood pressure of 24 to 48 hours' duration.

Chloroform Anesthesia. The dogs given a one-hour period of deep surgical anesthesia with chloroform also failed to exhibit a blood pressure fall. During the period of anesthesia, a blood pressure depression usually occurred. In every case, however, the pressures were restored to hypertensive levels within 24 hours and remained elevated subsequently.

Carbon Tetrachloride. The three animals receiving oral carbon tetrachloride (CCl_4) became severely ill; one succumbed, showing acute severe hepatic and (to a lesser degree) renal necrosis at post mortem. Rather than a blood pressure fall, however, these animals exhibited an exacerbation of their hypertensive state. Seventeen dogs were given CCl_4 either subcutaneously or intramuscularly. Five of these exhibited a slight and 2 a definite depressor response; the other 10 did not respond. No correlation could be established between the type of response and the parenteral path of administration. In most cases, subcutaneous or intramuscular CCl_4 produced a moderately severe local inflammatory reaction, with induration going on to fluctuation, but rarely to sloughing and ulceration. The local process ran its apparent course in 3 to 6 days, and was accompanied by fever (1 to 2°C. temperature elevation), moderate polymorphonuclear leucocytosis, increased sedimentation rate, decreased hemoglobin, anorexia and loss of weight.

Kidney Tissue Implantation. Subcutaneous implantation of 15 to 30 grams of fresh dog kidney tissue resulted in a definite sustained depressor response in about one-half the animals studied. This procedure led to the development of a local abscess, with subsequent disruption of the sutures, drainage and ulceration, going on to healing in 1 to 3 weeks. The systemic effects in all of these animals were essentially similar in duration and type to those observed with the CCl_4 abscesses.

Subcutaneous Turpentine. These injections produced a sustained depressor ef-

⁴ Repeated control pressures on Z39 averaged 175/105 mm. Hg. In view of the elevated diastolic blood pressure, this dog was tentatively classified as spontaneous hypertensive (12).

fect in a high percentage of animals. From the standpoint of positive depressor responses, it appeared to be the most effective agent. Subcutaneously injected turpentine produced a marked local inflammatory reaction, with abscess formation, sloughing and ulceration in 2 to 4 days, healing in 2 to 3 weeks. Systemic effects were similar in nature and duration to those noted with CCl_4 injection and subcutaneous kidney implantation.

Analysis of the data on dogs given CCl_4 , kidney, or turpentine failed to reveal any correlation between the nature of local and systemic reactions, and the presence or absence of a depressor response. Essentially similar responses were exhibited by

TABLE 1. RESPONSE OF SPONTANEOUS AND NEPHROGENIC HYPERTENSIVE DOGS TO TISSUE INJURY

TYPE OF MATERIAL ADMINISTERED	BLOOD PRESSURE RESPONSE			TOTAL NUMBER OF EXPERIMENTS
	None	Slight	Definite	
Subcutaneous (left flank) fresh dog kidney, 15-30 gm.	4	1	4	9
CCl_4 , Intramuscular (1 injection, left thigh, 5-10 cc.) or subcutaneous ($\frac{1}{2}$ cc. q.d. for 5-7 days)	10	5	2	17
Oral CCl_4 , 0.8 cc/kg.	3	0	0	3
Inhalation of chloroform to produce deep surgical anesthesia for 1 hr.	6	0	0	6
Subcutaneous turpentine, left axilla, 2.5 cc.	1	2	6	9
Intravenous typhoid pyrogenic vaccine, 100-150 million units in 4 hr.	5	0	0	5
Intravenous pneumococcus vaccine, 2 cc. (billion organisms) q.d. for 3-7 days	4	2	0	6
Intravenous staphylococcus vaccine, single injection, 2 cc. (billion organisms)	2	0	0	2

spontaneous and nephrogenic hypertensive dogs to the various types of tissue injury. The same agents elicited depressor responses in both types of hypertensive animals.

Renal Clearances. The serial changes in renal clearances and blood pressure following tissue injury of various types are summarized in table 2⁵. Analysis of these data from 12 experiments on 8 dogs reveals that the responses tend to fall into two general groups, based on type of blood pressure and renal flow response:

GROUP I. In 6 experiments (Z11-B, Z15, Z36, Z39, Z93-A, Z95) the dogs exhibited an early fall in blood pressure after tissue injury. In these animals, the depressor res-

⁵ In order to rule out the possibility that the changes in kidney function following tissue injury might be artefacts associated with frequently repeated clearance determinations over a short period of time, control clearances at 2 to 6 day intervals were done on a number of dogs. No significant changes were observed.

TABLE 2. SUMMARY OF RENAL CLEARANCE CHANGES FOLLOWING TISSUE INJURY

DOG NO.	TYPE & DURATION OF HYPERTENSION	WEIGHT	S.A. ¹	PROCEDURE	TIME INTERVAL, DAYS	B.P. ¹	NO. OF CLEARANCES	NO. OF PERIODS	RBF ¹	RPF ¹	GFR ¹	F.F. ¹
		kg.	M ²			mm.Hg			cc/min/ M ²	cc/min/ M ²	cc/min/ M ²	
Z11-B	S.H., ² 1038 days	18.8	.706	Subcut. turpen- tine	Control 1-4 ³	200/110 135/75	2 ⁴ 1	5 ⁴ 3	375 468	225 304	84 92	.373 .303
Z15	S.H., 822 days	14.5	.668	Subcut. re- nal tissue	Control 1-27 38th 73rd 75 et seq.	195/110 155/75 185/115 185/95 190/110	5 5 1 1 3	12 13 2 3 7	513 582 581 489 547	272 343 308 259 301	84 80 49 31 85	.308 .234 .127 .120 .282
Z36	N.H., ⁵ 557 days	20.8	.848	Subcut. re- nal tissue	Control 1-4 6-10 11 et seq.	185/110 160/85 190/115 190/120	3 2 2 1 ⁴	10 6 6 3 ⁴	399 505 275 385	211 293 176 212	68 74 60 64	.324 .254 .342 .302
Z39	S.H., 425 days	12.6	.610	Subcut. turpen- tine	Control 1-15 29th	175/105 140/80 165/90	1 1 1	3 3 2	536 624 407	284 374 236	87 89 85	.307 .238 .360
Z93-A	N.H., 180 days	14.2	.658	Intramusc. CCl ₄	Control 1-14 16-28 29 et seq.	200/110 180/90 190/105 195/110	3 4 2 7	8 12 6 15	453 600 574 490	240 378 344 260	84 84 84 84	.348 .222 .244 .323
Z95	N.H., 330 days	22.4	.892	Subcut. turpen- tine	Control 1-11 14th 28th 33rd	200/115 150/85 220/130 190/115 210/120	5 2 1 1 1	13 6 3 3 3	287 467 515 345 476	158 280 304 212 281	65 93 85 64 77	.411 .332 .280 .302 .275
Z11-A	S.H., 770 days	18.8	.706	Subcut. re- nal tissue	Control 1-9 13-17 18 et seq.	180/110 175/100 155/80 200/110	3 3 1 2 ⁴	8 9 3 5 ⁴	455 508 375	241 284 225	82 87 84	.339 .303 .373
Z14-A	N.H., 461 days	12.3	.599	Subcut. re- nal tissue	Control 1-4 5th 10th 13-20 21 et seq.	215/115 200/105 155/100 200/100 185/85 215/120	3 2 1 1 1 3	9 6 3 3 3 7	270 282 564 199 199 243	144 166 404 141 144 129	47 47 55 43 38 52	.314 .284 .135 .307 .268 .403
Z14-B	N.H., 765 days	12.3	.599	Subcut. turpen- tine	Control 6-30 48th	210/120 190/85 215/120	2 3 1	5 9 2	298 296 313	164 157 172	65 50 48	.397 .318 .279
Z20	S.H., 765 days	32.4	1.131	Subcut. re- nal tissue	Control 1-5 8-15 20 et seq.	195/115 195/105 165/85 200/110	5 2 2 5	13 6 6 13	534 539 442 566	294 313 247 300	100 98 78 96	.340 .314 .317 .320
Z93-B	N.H., 275 days	14.2	.658	Subcut. re- nal tissue	Control 1-12 16 et seq.	215/115 200/110 200/110	1 2 1	2 6 3	469 430 430	258 258 258	68 85 85	.264 .330 .330
Z93-C	N.H., 325 days	14.2	.658	Intramusc. CCl ₄	Control 1st 3rd 6th et seq.	200/110 160/105 140/105 185/110	3 1 1 2	9 2 2 4	430 453 520 447	258 249 307 246	85 100 84 82	.330 .438 .274 .333

¹ S.A.: Surface area = $\frac{1.13 \times W^{.67}}{10,000}$, where W is weight in gm. B.P.: Blood pressure. RBF: Renal blood

flow. RPF: Renal plasma flow. GFR: Glomerular filtration rate. FF: Filtration fraction. ² S.H.: Spontaneous hypertensive. ³ Dog succumbed during surgical procedure on the following day. ⁴ Clearance done with dog anesthetized with sodium pentobarbital, approximately 25 mg/kg. ⁵ N.H.: Nephrogenic hypertensive. ⁶ See text.

ponse was noted 24 hours after the injurious stimulus and persisted for 4 to 27 days. It was accompanied in every case by a significant sustained increase in renal plasma and blood flow (table 2)⁶. In 3 animals of *Group I* (Z15, Z93-A, Z95) the RPF and RBF remained elevated above control levels for a variable time, up to 14 days, after the blood pressure had returned to hypertensive levels.

In this group, 3 dogs were injected with turpentine, one with CCl₄ and 2 were implanted with renal tissue. Of these 6 animals, 3 were spontaneous and 3 were nephrogenic hypertensives. The 3 spontaneous hypertensive dogs showed control RPF and RBF values within normal limits for this species (12, 13)⁷. Control RPF and RBF data on the 3 nephrogenic hypertensives revealed the following: Z93-normal; Z36-low normal; Z95-significantly reduced below normal. These 6 animals all responded

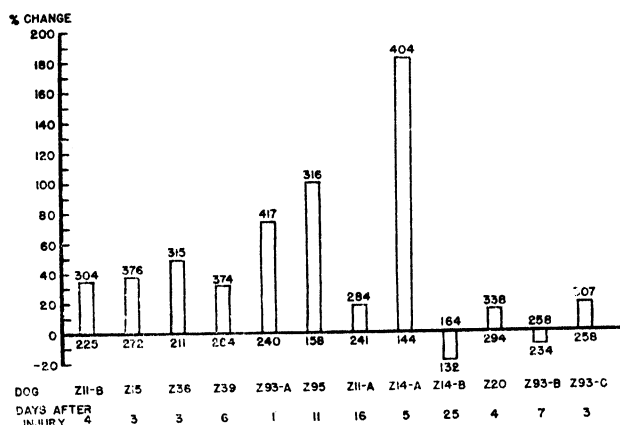


FIG. 1. MAXIMUM PERCENTAGE CHANGES in renal plasma flow following tissue injury to spontaneous and nephrogenic hypertensive dogs. All 12 experiments are summarized. Actual values for the various functions are indicated; the control values are recorded along the 0% axis, the values at the time of maximum change in renal plasma flow are recorded at the top or bottom of the column for each dog indicating the percentage change. *Days after injury* indicates the time at which the maximum RPF changes from control values were observed.

to abscess with an increase in RPF and RBF of such degree as to raise these functions considerably above average normal values for the dog. The maximum changes in renal plasma flow after abscess production are presented graphically in figure 1. For this group, the maximum percentage increases in RPF ranged from 21 to 100 per cent. Dog Z95, with control clearances depressed considerably below normal, showed the greatest percentage increase (100%) and attained a maximum RPF level of 316 cc/min/m². This value is in the range reached after injury by other dogs with normal control clearances. It is to be noted that in this group, an early and sustained depressor response, accompanied by a persistent renal hyperemia, was observed after tissue injury.

⁶ The RPF exhibits a greater increase than the RBF due to the development of a moderate anemia with tissue injury.

⁷ The renal clearance data on these spontaneous and nephrogenic hypertensive dogs, and on the normotensive dogs studied in this laboratory, are presented in detail in a previous paper (12).

GROUP II. By contrast, in the 6 other experiments (*Z11-A*, *Z14-A*, *Z14-B*, *Z20*, *Z93-B*, *Z93-C*) neither a sustained depressor response nor a persistent renal hyperemia was seen in the first few days after injury. In 2 cases (*Z93-B*, *Z93-C*), tissue injury failed to elicit any depressor response, early or late. In these 2 experiments no renal clearance changes were noted with the exception of a single observation (3rd day, *Z93-C*). Four animals (*Z11-A*, *Z14-A*, *Z14-B*, *Z20*) exhibited a *late* depressor response to tissue injury, with the onset of the blood pressure fall 6 to 8 days after injury. Their reduced arterial pressures persisted 4 to 30 days. During this period these animals presented varied renal plasma and blood flow findings (table 2). *Dog Z11-A* exhibited a moderate renal hyperemia. *Dogs Z14-A* and *Z20* had reduced renal plasma and blood flow. *Dog Z14-B* showed no clearance alterations.

One of the nephrogenic hypertensive dogs in this group, *Z14*, had the lowest control RPF and RBF in this series. The clearance done on *Z14-A* on the 5th day following injury revealed a marked elevation in flow, from a control RPF of 144 to 404 cc/min/m², one of the highest flows recorded. This represents an increase of 181 per cent, the largest observed in this study (fig. 1). There was no significant fall in blood pressure at this time.

Dog Z11-A exhibited an unusual phenomenon: Three clearance determinations (9 periods) during the first 6 days following subcutaneous kidney implantation revealed an effective RPF of 46, 50, 45 with a GFR of 54, 80, 52 cc/min/m². This paradoxical situation is apparently due to an unexplained interference with the tubular mechanism for the excretion of PAH (14, 15). Here the PAH clearance cannot be considered an index of the true renal plasma flow.

In all 12 experiments, there were no significant changes in GFR accompanying either a fall in blood pressure or an increase in RPF. Hence, in those dogs exhibiting an increase in RPF, there was a significant reduction in the filtration fraction, indicating decreased efferent arteriolar resistance (9-11, 16-18). In *dog Z15*, a significant reduction in GFR, of unexplained origin, was observed in clearance studies on the 38th and 73rd days following tissue injury (table 2).

In the 11 experiments in which the dogs survived, renal clearance values as well as blood pressures eventually returned to control levels (table 2).

DISCUSSION

Nature of the Renal Hyperemia. Our data demonstrate *conclusively* that a single tissue injuring stimulus may elicit a sustained increase in renal plasma flow and renal blood flow in hypertensive dogs, persisting for as long as 28 days. It has been shown (9-11, 16, 17) that pyrogenic inulin or typhoid vaccine produce an acute renal hyperemia in normotensive and hypertensive patients or dogs, beginning about 1½ hours after injection and persisting for 2 to 3 hours. Page and co-workers (9, 19-22) have reported improved kidney function, including increased effective renal blood flow, accompanying blood pressure reduction in hypertensive dogs and humans after the injection of renal and other tissue extracts. More recently, these workers (23) reported initial moderate depression of renal function, followed by an eventual restoration to or above normal levels, in cases of malignant hypertension treated with bacterial products.

Both spontaneous and nephrogenic hypertensive dogs may respond to tissue injury with a marked sustained renal hyperemia, despite a concomitant fall in systemic arterial pressure. The increase in RBF occurring spontaneously with a blood pressure fall confirms previous data (14, 15, 24) suggesting that the kidney is able to maintain its circulation in the face of marked fluctuations in blood pressure.

It is further apparent that in dogs with partial ligation of the renal arteries leading to a reduction in effective renal blood flow, mechanical narrowing of the main renal arteries by ligatures is not the sole factor determining the upper limits of renal flow. The ability of such animals to develop a marked prolonged renal hyperemia demonstrates that reversible dynamic vasoconstriction, rather than anatomically fixed vascular obstruction, accounts in part for the decreased renal blood flow. In this respect the nephrogenic hypertensive dog with diminished renal blood flow closely resembles man with essential hypertension, who also responds to 'pyrogens' with renal hyperemia (9-11, 16, 17).

The observed increase in renal blood flow, with little change in glomerular filtration rate, is possible only on the basis of a predominant decrease in efferent arteriolar resistance. A similar conclusion results from an analysis of the effect of 'pyrogens' on renal circulation in human hypertensives (9-11, 16, 17). The development of renal hyperemia may depend upon autonomous intrarenal mechanisms or may result from a general systemic response mediated by humoral or nervous pathways. Evidence has accumulated indicating that the nerves to the kidney are probably not essential for a hyperemic response (9-11, 15-18, 25).

It is now known that a number of apparently unrelated agents elicit an increased renal blood flow. Thus, intravenous pyrogen, amino acids (26), adenosine derivatives (27) or oral proteins (26) all produce renal hyperemia. These may also act as systemic vasodilators.

Further, various hormones are capable of eliciting a prolonged increase in renal plasma flow in the normal dog. Among these are thyroid, whole anterior pituitary extract, purified pituitary growth hormone, desoxycorticosterone acetate and possibly purified pituitary adrenocorticotrophic hormone (ACTH) (28-31). Tissue injury, including subcutaneous injections of chemical irritating agents, has been demonstrated to be an alarm stimulus that causes increased release of adreno-corticoids, the response being mediated by ACTH (32-36). Recently Corcoran and Page (37) reported that pyrogens cause a significant increase in the urinary excretion of corticosteroid-like substances in patients with malignant hypertension.

Finally, ACTH injection, by stimulating and altering the pattern of adrenal corticoid secretion, leads to protein catabolism and negative nitrogen balance (38-40) possibly releasing into the blood stream metabolites affecting renal vascular resistance (26, 27, 41-43). Thus the possibility exists that the complex of metabolic and hormonal changes occurring with tissue injury may play an important role in the production of the renal hyperemia.

Nature of the Blood Pressure Fall. In the present study, subcutaneous injection of turpentine was the form of tissue injury most effective in eliciting a sustained depressor response. It is apparent that the blood pressure reductions elicited by this agent, and by carbon tetrachloride, are *nonspecific* with respect to presumed renal

hypertensive mechanisms. In all likelihood, the depressor response following kidney implantation is also nonspecific.

The data from present and previous (5, 6), studies indicate that only certain types of tissue injury provoke a sustained fall in blood pressure. Thus, oral carbon tetrachloride or inhaled chloroform in large doses either have no effect on the blood pressures of hypertensive dogs, or may even cause a further rise in pressure. Both these agents cause liver injury (necrosis), without localized abscess formation. In this regard, it is noteworthy that hypertensinogen is produced in the liver and that hepatic damage by carbon tetrachloride leads to a progressive diminution in the blood concentration of hypertensinogen until it finally disappears (44). Presumably this occurred in our dogs given carbon tetrachloride, since liver damage was severe enough to kill one of the 3 animals. Nevertheless, a pressor, rather than depressor, response was observed. It is possible that this effect may result from a reduction in the ability of the liver to inactivate pressor steroids such as desoxycorticosterone.

There appears to be one common factor in the responses to the 3 agents in the present study provoking a fall in blood pressure, namely, the production of a marked local and systemic reaction, with formation of an initially sterile (carbon tetrachloride, turpentine) or non-sterile (kidney tissue) abscess. However, it is noteworthy that despite abscess formation and an apparently similar systemic reaction, hypertensive dogs previously studied failed to develop a depressor response to several other materials implanted subcutaneously (e.g. fresh dog heart muscle or liver, egg yolk, infusorial earth) (6). Moreover, a significant number of dogs in the present series failed to respond with a blood pressure fall to turpentine, carbon tetrachloride or kidney implantation, although they developed an apparently similar pattern of local and systemic reactions. These discrepancies cannot at present be correlated with any other observations made on these animals.

The time relations between the implantation of material, the duration of the local and systemic reactions, and the duration of the blood pressure fall make it clear that the various agents utilized are not depressors or vasodilators per se. It is more likely that the depressor effects are the end-results in the vascular bed of a general systemic reaction to the 'pyrogenic' materials. Bradley (17) has suggested that a reduction in peripheral resistance due to arteriolar dilatation is the primary hemodynamic change leading to a fall in blood pressure following 'pyrogens.' Data from this laboratory (45) lead us to agree with this concept.

It is possible that the *general* arteriolar dilatation ensuing after tissue injury follows upon and results from a renal hyperemia induced primarily. According to this hypothesis, the renal hyperemia would act to correct the disturbed kidney physiology and thereby eliminate a renal pressor mechanism. However, evidence against this concept is the fact that in several of our dogs the renal hyperemia persisted for a period after the blood pressure had returned to hypertensive levels. Further, in some dogs the depressor response was either unassociated with or was asynchronous with the renal hyperemia. It seems more likely that the decreases in both renal and generalized vascular resistance are the common product of the systemic reaction to injury. This arteriolar vasodilatation may result from a direct response of the autonomic nervous system to tissue injury. It may be brought about by the release of vaso-

dilator materials from the abscess site, or by the intermediation of other humoral mechanisms. Tissue injury is known to be a stimulus for an alarm reaction, eliciting alterations in pituitary adrenocorticotrophic and adrenal corticoid secretion. The resulting effects upon protein, carbohydrate, and mineral metabolism, as well as upon vascular reactivity, may be of prime importance in the genesis of the vasodilatation which results in the return of the blood pressure to normal values.

SUMMARY

The effect of tissue injury on blood pressure and on renal clearances was studied in nephrogenic and spontaneous hypertensive dogs. Local parenteral injections of turpentine or carbon tetrachloride, or the subcutaneous implantation of kidney tissue, resulted in the production of an abscess and a sustained fall in blood pressure in both nephrogenic and spontaneous hypertensive dogs. This was associated with an increase in renal blood flow. Since the chemical agents are equally effective in eliciting the blood pressure fall, it is concluded that the depressor response to tissue injury is a non-specific reaction.

Injury produced by inhalation of chloroform or by oral administration of carbon tetrachloride consistently failed to reduce the blood pressure of hypertensive dogs. Instead, the blood pressure was sometimes elevated. The mechanism involved in the fall in blood pressure, therefore, appears to be part of the pattern of systemic reaction associated with inflammation (abscess). Abscess production elicits a persistent increase in renal blood flow. However, the renal hyperemia is sometimes asynchronous with the blood pressure response. The reduction in blood pressure is therefore not directly dependent upon 'relief of renal ischemia.'

Nephrogenic hypertensive dogs, with and without a reduced renal blood flow, showed as ready a capacity to respond to tissue injury with a renal hyperemia as did spontaneous hypertensive animals. It is concluded that dynamic vasoconstriction plays a role in the impaired renal circulation observed in some nephrogenic hypertensive dogs. Pathogenic implications of these data are further discussed.

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IMPORTANCE OF DIETARY PROTEIN, CALORIES AND SALT IN EXPERIMENTAL RENAL HYPERTENSION^{1, 2, 3}

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THE data to be presented were obtained in the course of control experiments preliminary to studies of the significance of liver function in 'renal' hypertension. At the time this work was inaugurated, a survey of the literature revealed that no definitive studies of the influence of diet in experimental renal hypertension, involving sufficient numbers of animals, had yet been reported. The large body of controversial literature on the effects of diet in human hypertension will not be considered here.

Relatively few investigators had employed rats in such studies and none of these had used the 'synthetic' rations which may now be employed with this species. Thus, Chanutin and his co-workers (1) observed renal hypertrophy in normal rats on high meat diets but no hypertension. Their observations are in accord with those of several other laboratories (2-5). However, Chanutin *et al.* (1) did observe elevated blood pressures in rats fed high meat diets following subtotal nephrectomy. Martin (6) has reported hypertension in rats fed diets containing 5 to 10 per cent of tyrosine while Grollman and Harrison (7) have demonstrated a return to normal pressures in renal hypertensive rats ingesting a stock diet which had been dialyzed free of its diffusible components. Further, a return to hypertensive levels was observed upon addition of NaCl but not of KCl to this ration.

That high protein diets induce renal changes in the rabbit has been amply demonstrated (8-12). However, only one group reported hypertension in such animals (11) while another laboratory observed no hypertension even after reduction of renal mass (12). The effects of dietary protein and salt in dogs with renal ischemia or silk-wrapped kidneys also appears to be controversial. Elevation in pressure has been reported after feeding meat (13-15), salt (13) or urea (13, 15). Contrary findings have been somewhat more numerous (16-21).

In 1946, after reviewing these findings, Braun-Menendez *et al.* (22) concluded, "... it has not been definitely demonstrated that diets rich in protein are capable of exaggerating renal hypertension. Taking into account, however, the importance of the kidney in the elimination of products or protein catabolism, it is possible that these diets modify to some extent the evolution of renal hypertension."

The present paper provides evidence establishing the significance of at least three dietary factors, calories, protein and salt, in the behavior of the systolic blood pressure of rats following subtotal nephrectomy.

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EXPERIMENTAL

The rats employed in these experiments were males of the Vanderbilt and Carworth strains. Hypertension was produced by a modification of the two-stage process of Chanutin and Ferriss (23), performed when the rats had attained a weight of 200 gm. In the first stage a figure-of-eight ligature was applied to the left kidney under sodium pentobarbital anesthesia. One week later the right kidney was removed. By leaving somewhat more renal tissue than is usually done we obtained a less severe hypertension than in our previous work (30) but lost fewer rats in acute renal insufficiency. Systolic pressures were determined using an improved model of the Chittum, Hill and Grimson (25) modification of the end-point device of Skeggs and Leonards (24). The commercial 'chow' used was obtained from the Ralston-Purina Co., St Louis, Missouri. The composition of the synthetic diets is summarized in

TABLE 1. COMPOSITION OF DIETS

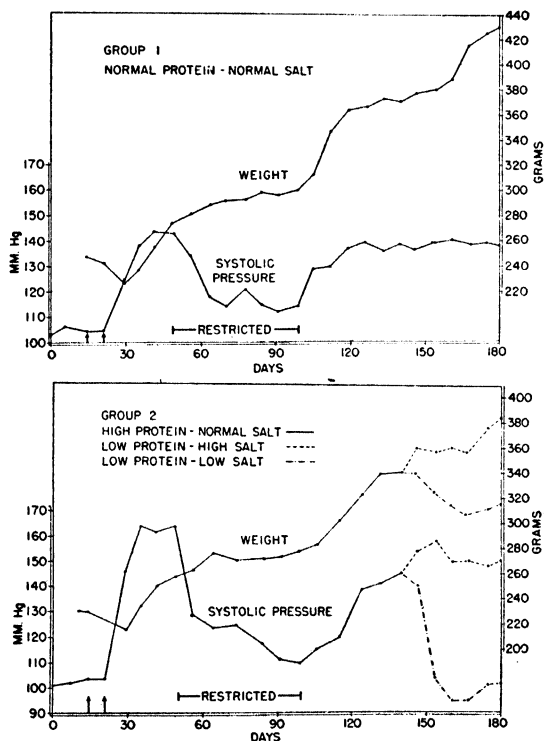
	1	2	3	4	5	6	7	8
Casein	18	50	8	8	8	50	8	8
Sucrose	61	29	71	71	68	29	71	68
Cotton oil	10	10	10	10	10	10	10	10
Crisco	5	5	5	5	5	5	5	5
Cod liver oil	2	2	2	2	2	2	2	2
Salts-A	4	4	4		4		4	4
Salts-B				4		4		
Choline			0.3	0.3	0.3		0.3	0.3
Cystine			0.3	0.3	0.3		0.3	0.3
NaCl					3.0			
Urea							12	
Phenylalanine								1.5
Tyrosine								1.5

To each kilogram of mixed diet were added thiamine 5 mg., riboflavin 10 mg., pyridoxine 5 mg., calcium pantothenate 25 mg., niacin 75 mg., inositol 50 mg., 2-methyl-1,4-naphthoquinone 5 mg., *p*-aminobenzoic acid 100 mg., and α -tocopherol 25 mg. In addition each rat received 150 γ per week of biotin and folic acid.

table 1. Salt mixture A was that of Hubbell, Mendel and Wakeman (26) which contains 11.2 per cent KCl and 6.9 per cent NaCl. The NaCl was omitted from salt mixture B. Water was offered *ad libitum* and the animals were housed in individual cages with wire mesh bottoms.

The rats were offered the various experimental rations for 3 weeks before surgery and maintained on the same rations thereafter. Repeated measurements of blood pressure were made during this period. This served both to establish a baseline for the experiment and to accustom the animal to the routine procedure for estimation of blood pressure. Consistent values were obtained after the third determination. During the first 14 days of this period, the mean daily consumption of the rats on the various low protein diets was about 1.1 gm. less than that of the high protein-fed rats. Thereafter, all rats were offered only the amount ingested *ad libitum* by the rats on the low protein diets. Within 5 weeks after surgery they stabilized in

pressure. At this time the daily food consumption of each group but *A* was restricted to 66 per cent of the previous daily consumption. This was continued for 5 weeks, after which the animals were allowed to eat *ad libitum* of the same diets they had been offered previously. For about 10 days they ate about 50 per cent more than their previous *ad libitum* level, then returned to this level over a period of about one week. Five weeks after the food restriction was removed, each group was divided into two subgroups and offered the standard amount of each of two different diets as indicated in the figures. This was continued for 5 weeks. At this time, blood was taken for

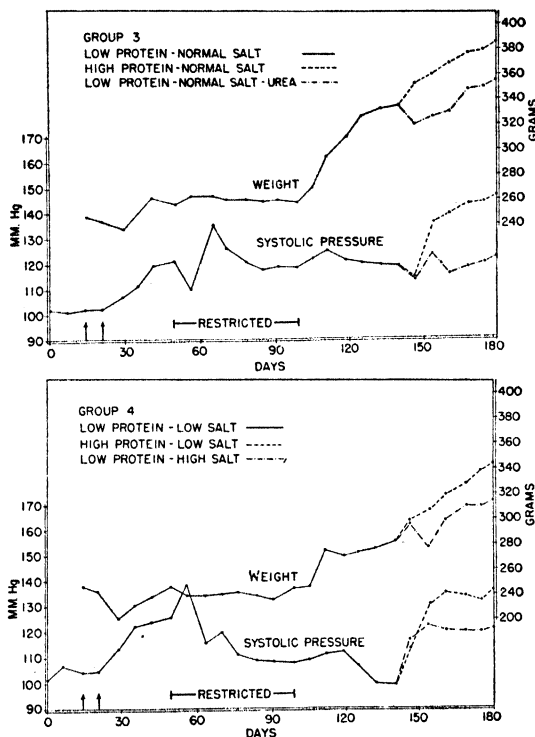


Figs. 1 and 2. RELATIONSHIP between diet and blood pressure

NPN determinations and the experiment terminated. The results are shown in figures 1 to 8. Two weeks after surgery, each group contained at least 17 rats. At the end of the experiment, no group contained less than 15 rats.

Figure 1. The animals on our 'mock chow' ration no. 1 developed a moderate hypertension after surgery. Food restriction in this group resulted in a fall and stabilization of pressure at levels only slightly above normal, while still permitting a moderate weight gain. When the food restriction was lifted the animals gained weight rapidly and pressures returned to their original levels over the course of 2 weeks and remained so for the duration of the experiment.

Figure 2. Ingestion of high protein, normal salt diet 2 resulted in stabilization of systolic pressures at about 165 mm. before food restriction. The diminished food consumption in this group resulted in a precipitate fall to about 130 mm. with a gradual decline to 110 mm. although the total food consumption still supported very slow growth. When the rats returned to *ad libitum* feeding, excellent growth followed and systolic pressures returned to somewhat less than the pre-restriction level over the course of 5 weeks. Subgroup 2a was then switched to low protein, low sodium diet



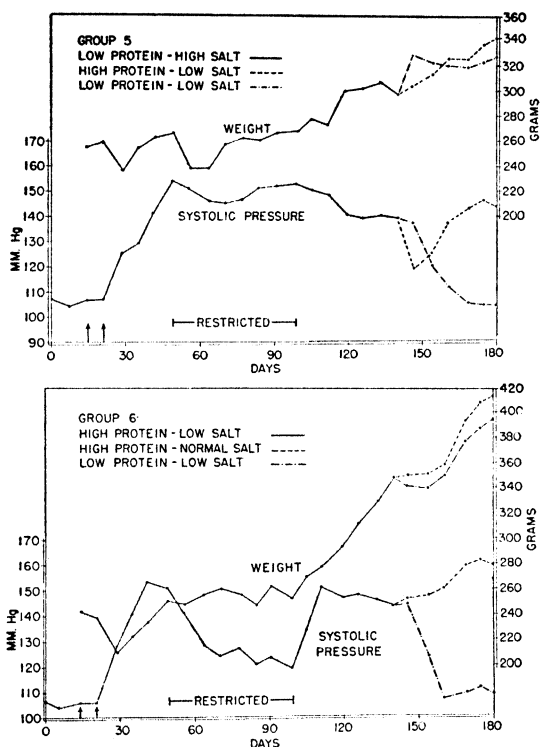
Figs. 3 and 4. RELATIONSHIP between diet and blood pressure

4. Their pressures dropped to normal values within 2 weeks. This was accompanied by a moderate weight loss. In contrast, subgroup 2b which was switched to low protein, high sodium diet 5, actually showed a rise in pressure of about 10 mm. in the first 2 weeks followed by a fall to the original level.

Figure 3. Systolic pressures in the animals on low protein, normal salt diet 9 were about 118 mm. before food restriction. In the ensuing 5 weeks there were some variations but at the end of this period, the mean blood pressure was 122 mm. In this group, as in the other groups on low protein rations (see fig. 4, 7) food restriction resulted in an actual rise in blood pressure, sometimes after a week's delay, but invariably this was followed by a fall to a low level. During this period the rats' weights remained surprisingly constant. In the 5 weeks of *ad libitum* feeding which followed, the rats gained an average of 75 gm. each, but with no significant alteration in pres-

sure. *Subgroup 3a* was then given high protein, normal salt diet 2 and this resulted in a rise of 35 mm. in 5 weeks. To the diet of *subgroup 3b* was added urea in the amount expected from the metabolism of the protein in the diet of *group 3a*; this resulted in a negligible rise in pressure even after 5 weeks.

Figure 4. Virtually no hypertension was seen during the basal period in the animals on low protein, low salt ration 4. Food restriction elicited a temporary rise in pressure of about 18 mm. which subsided within 2 weeks so that at the end of

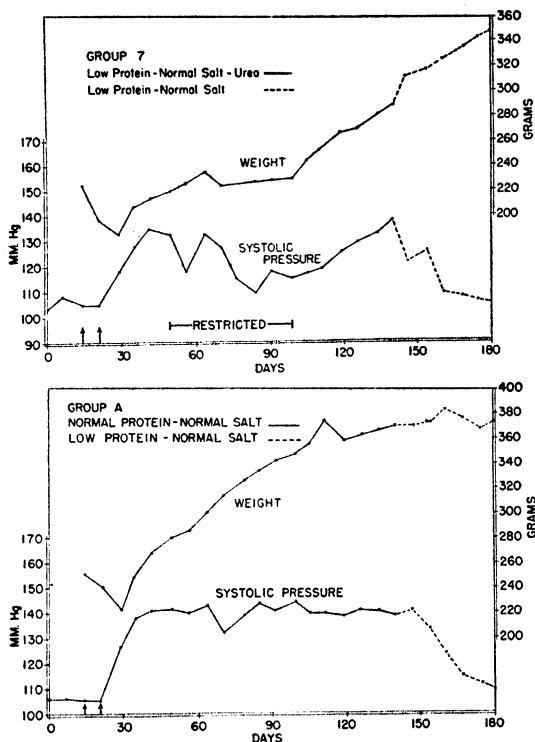


Figs. 5 and 6. RELATIONSHIP between diet and blood pressure

the restriction period the mean pressure of this group was 112 mm. and they had lost an average of 18 gm. in weight. While *ad libitum* feeding permitted a steady increase in weight, the mean systolic pressure of this group fell 12 mm. during the period of realimentation. At this time, to the diet of *subgroup 4a* was added 3 per cent NaCl so that they were then receiving low protein, high salt diet 5. This resulted in a rise in pressure of 24 mm. within 3 weeks; the maximum attained level was 125 mm. *Subgroup 4b* was transferred to diet 6, the high protein, salt poor ration. The rise in pressure was even more dramatic than that elicited by the addition of salt.

Figure 5. A low protein, high salt diet 5 was offered to *group 5*. Uniquely, in this group systolic pressure continued to rise during the restriction period and stabilized at 145 to 155 mm. These rats lost some weight in the first 2 weeks of restriction

but gained slowly, thereafter. On return to *ad libitum* feeding, the growth rate increased but was slower than that observed on the low protein, normal salt ration. Moreover, there occurred an average fall of 12 mm. in pressure during this period. *Subgroup 5a* was transferred to low protein, low salt diet 4 and, after a one-week lag period, their pressures fell sharply. Blood pressures of *subgroup 5b*, which was offered high protein, low salt ration 6, fell by 20 mm. in the first week as the low salt effect proceeded. Thereafter, however, the high protein effect was dominant and blood pressures in this group increased rapidly.



Figs. 7 and 8. RELATIONSHIP between diet and blood pressure

Figure 6. A high protein, low salt diet 6 was initially offered to group 6. Pressures in this group did not quite attain the level seen in the high protein, normal salt group 2 before restriction. Yet, in contrast, during restriction they did not fall quite as precipitately as did group 2. Upon removal of the food restriction these rats gained weight rapidly and their pressures rose to levels comparable to those of the high protein, normal salt-fed animals at this time. Transfer to the latter ration did, however, result in a slow rise of 16 mm. Switching subgroup 6b to low protein, low salt ration 4, on the other hand, resulted in a sharp drop in pressure after one week.

Figure 7. In the period before restriction, pressures in group 7, receiving the low protein, normal salt diet plus urea (diet 7), stabilized at a level slightly higher

than that of the rats on low protein, normal salt ration 3. Their pressures returned to basal levels during restriction after the usual temporary rise associated with low protein diets. On return to *ad libitum* feeding there occurred a steady rise in blood pressure which, however, was neither as rapid as that seen in animals on high protein, normal salt ration 2, or high protein, low salt ration 6 nor did it attain the same height. That this rise was due entirely to the presence of urea was demonstrated by the fall in pressures which occurred when the urea was removed from the diet of this group.

Figure 8. Group A was maintained on 'mock chow' ration 1 but was never restricted and served as a control on all other groups through this period. Their pressures remained stable at about 140 mm. After 17 weeks the entire group was transferred to low protein, normal salt ration 3 and, after a lag of one week, pressures fell steadily, reaching 112 mm. in 4 weeks.

TABLE 2. BLOOD NPN LEVELS AT TERMINATION OF EXPERIMENT

Number	DIET		SUBGROUPS	NPN mg. %
	Protein	Salt		
1	Normal	Normal	1a	47.2
2	High	Normal	3a, 6a	52.4
3	Low	Normal	Aa, 7a	37.6
4	Low	Low	2a, 5a, 6b	40.7
5	Low	High	2b, 4a	26.7
6	High	Low	4b, 5b	45.5
7	Low	Normal + Urea	3b	44.7

At the termination of the experiment, blood samples were obtained by heart puncture. The results of NPN determinations performed on these samples are summarized in table 2. Despite previous differences in history, excellent agreement was found between the NPN values observed in rats from different subgroups but which had been ingesting the same diet for the last 5 weeks of the experiment. Consequently, the values quoted in the table are the means for all rats on the same diet during the last part of the experiment. The source of these animals, and, therefore, their previous history, is given in the table for reference. In general, it will be seen that elevated NPN values were found in all but one group. There was a simple correlation between dietary protein and blood NPN; the higher the dietary protein level had been, the higher the NPN level, all other things being equal. The presence or absence of dietary salt at any given protein level was of little significance. However, the addition of unusually large quantities of salt in diet 10 apparently resulted in a diuresis sufficient to maintain normal NPN values despite the markedly reduced renal mass.

DISCUSSION

The data obtained in this study leave little doubt that the systolic blood pressure of rats with hypertension induced by subtotal nephrectomy may be influenced by at least three dietary factors, namely, caloric intake, protein and sodium. We have interpreted the fall in blood pressure which accompanied dietary restriction as being

due largely to caloric restriction as it was observed on virtually all our dietary regimes. This is in accord with a considerable body of clinical observations and particularly with the report of Brozek, Chapman and Keys (27), and the disappearance of hypertension in besieged Leningrad (28). In contrast to the latter situation, however, realimentation of the food-restricted rats of this study did not result in a rapid and violent rise in blood pressure but rather to a slow increase to levels which were usually somewhat below those found before imposing the food restriction. No obvious explanation is at hand for the transient rise in blood pressure observed during the restricted feeding of rats on low protein rations. It may be that it is associated with a temporary period of negative nitrogen balance with 'catabolism' of appreciable quantities of the rat's own protein so that the animal may be regarded as being, temporarily, on a high protein diet. The restriction of food intake which was arbitrarily used in this study, while effective in lowering blood pressure, was not severe enough to cause a loss of body weight. In fact, most of the animals actually gained weight slowly throughout this period. It is difficult to translate this into human terms since, unlike man, the adult rat continues to gain weight and remain in positive nitrogen balance.

The significance of dietary protein in the maintenance of systolic blood pressure was demonstrated in our earlier studies (30) and amply confirmed in the present work. Only when the diet contained an overwhelming excess of salt was hypertension observed in rats on low protein rations. Again, however, the mechanism by which protein exerts its effects is unexplained. While urea feeding did elicit a rise in pressure, the effect was never as dramatic as that resulting from an equivalent protein intake. It is unlikely, then, that the effect of dietary protein on the blood pressure of rats in these experiments can be attributed merely to the increase in the osmotic work necessary to excrete the urea metabolized on a high protein diet. Indeed, it is equally possible that the comparatively minor effect observed after addition of urea to a low protein ration may be the result of somewhat increased protein synthesis in the rat utilizing the nitrogen of urea. This would be compatible with the observation that dietary urea accelerates the growth of young rats on low protein rations (29). While the data are not included in the figures, it should be noted that no rise in pressure was observed after the addition of large amounts of tyrosine and phenylalanine (diet 8) to a low protein ration. Further study of the nature of the protein effect is now in progress.

This study of the effects of salt on the blood pressure of hypertensive rats, made with the use of synthetic diets, has confirmed and extended the results of Grollman and Harrison (7) who used a dialyzed commercial chow. Virtual elimination of sodium from the diet of animals on low protein rations lowered systolic pressure to actually subnormal values. On the other hand, removal of sodium from the high protein diet only slightly reduced blood pressure. In like fashion, addition of excess salt to a low protein ration produced an impressive hypertension which was not reduced by caloric restriction. This phenomenon, in all likelihood, has no human analogy. The sodium chloride added to this diet was ten times the amount present in the basal salt mixture. Restriction to 66 per cent of this extremely high level, therefore, still permitted the ingestion of an apparently overwhelming salt excess. It should be noted, however,

that the ingestion of this high salt, low protein diet elicited a rise of but 5 to 10 mm. in pressure in 5 out of 8 control unoperated rats, and no rise in the remaining 3 animals.

An important difference in the behavior of the animals on high salt and on high protein rations should be noted. Regardless of the dietary salt level, the rats on high protein rations were clean and firm with their coats and tails in excellent condition. They were relatively fierce and aggressive animals which were active in their cages at all times. In contrast, the rats on low protein rations were dirty; their tails developed ulcers and were occasionally sloughed off. They were invariably sluggish animals, slow to respond to all stimuli. This picture was exaggerated by salt deficiency but was not altered by the incorporation of 3 per cent NaCl in the ration even though this did result in the development of hypertension. Because of these differences in behavior, on two occasions blood pressure measurements were made under light pentobarbital anesthesia. The data so obtained was essentially identical to that obtained on the same rats without anesthesia.

At this time it cannot be stated with any certainty whether hypertension, created in this fashion, bears analogy to any type of hypertensive disease known to occur in man. Studies of the influence of these dietary factors in other types of experimental hypertension in the rat are currently in progress. In any case, if the results of this study are transferred to the management of human hypertensive disease, only the qualitative concepts, not the quantitative data may be justifiably utilized or exploited.

SUMMARY

Purified, synthetic diets have been used in a study of the effects of various nutritional factors on the blood pressure of adult male rats rendered hypertensive by sub-total nephrectomy. It has been found that the dietary levels of protein, calories, and salt independently exert a profound influence on systolic blood pressure.

The effect of protein was determined at three dietary levels, all of which supported rat growth under these conditions. In rats ingesting diets of normal salt content, systolic pressures stabilized at the following levels: high protein, 168 mm.; medium protein, 145 mm.; low protein, 122 mm. While urea, added to a low protein ration, exerted a slight pressor effect, it was not sufficient to account for the observed effects of dietary protein. The addition to a low protein diet of tyrosine and phenylalanine, in the amounts present in a 50 per cent casein diet, had no effect on blood pressure.

Restriction of daily food consumption, to an amount just adequate to permit weight maintenance or very slow growth, resulted in a fall to virtually normal pressures on all diets but that containing an unusually excessive amount of salt. Drastic reduction of the sodium content of the diet only slightly reduced the systolic pressures of rats on high protein rations. The addition of 3 per cent NaCl (ten times the normal level) to a low protein ration resulted in marked hypertension.

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FAILURE OF PORTAL SYSTEM TO INACTIVATE COMPLETELY OVARIAN SECRETIONS IN IMMATURE RATS¹

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BY VARIOUS means, such as implantation of ovaries and estrogen pellets in the liver, spleen and mesentery, evidence has been accumulated that the portal system is a focus of estrogen degradation (1). Clinical material showing gynecomastia in human males coincident with chronic hepatitis has been interpreted as indicating that the human, like the laboratory animal, degraded estrogens in the liver. Hooker, Drill and Pfeiffer (2) have reviewed the material prior to their work on the monkey. Contrary to expectation, when estradiol and estrone pellets were implanted in the monkey's spleen the experimental animals responded as well as the control.

The findings on the monkey have reopened the question of the liver's capacity to degrade estrone. It appeared to us that it would be of interest to examine the capacity of the immature rat's liver to degrade estrone. A review of the literature revealed that all reported experiments had employed animals past the onset of puberty.

METHODS

Immature rats 40 ± 7 days of age were segregated into 3 groups of 25 each. *Group 1* was ovariectomized. Portions of each rat's ovarian tissue were teased into small pieces and placed in a large bore hypodermic needle. Insertion of the needle into the animal's spleen and the subsequent concomitant withdrawal of the needle and forcing out of the tissue with a plunger, served to produce a line of possible implantations along the site of the insertion. *Group 2* was ovariectomized but received no ovarian grafts. *Group 3* was unoperated. The animals were weighed at the start of the experiment and at frequent intervals thereafter for 54 days. The last weight was taken on the day of autopsy.

At autopsy each animal which had had tissue transplanted to the spleen was carefully examined for 1) ovarian tissue in the peritoneal cavity and 2) for any sign of adhesion to the spleen which might carry collateral circulation. The ovariectomized animals were checked for residual ovarian tissue.

The uteri of all animals were dissected out from the cervix to the tubal end. Uteri (with the exception of 4 from rats of the transplanted series) and adrenals were

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weighed. Uteri and grafted ovarian tissue were preserved from all transplanted animals and sample tissue from the ovariectomized and control group was also taken. Vaginal smears were taken at autopsy on the transplanted animals and the normal controls.

TABLE 1. INITIAL AND FINAL BODY WEIGHTS, UTERINE AND ADRENAL WEIGHTS OF EXPERIMENTAL AND CONTROL ANIMALS¹

TYPE OF ANIMAL ²	AVERAGE INITIAL WT.	AVERAGE FINAL WT.	AVERAGE WT. GAIN	AVERAGE UTERINE WT.	AVERAGE ADRENAL WT.
	gm.	gm.	gm.	mg.	
Unoperated control (no. = 23).....	46.7	165	113	278.1	38.1
Ovariectomized (no. = 16).....	47.1	189	139	33.3	42.4
Ovaries transplanted into spleen—no adhesions (no. = 10).....	60.2	182	122	72.3 ³	39.2
Ovaries transplanted into spleen—adhe- sions present (no. = 11).....	60.2	173	112	180.3 ³	38.4

Significant Differences and Probability Levels

COMPARISON BETWEEN	DIFFERENCE	t VALUE FOUND	DEGREES OF FREEDOM	PROBABILITY LEVEL	t VALUE AT LOWER P LEVEL
Wt. gain of ovariectomized animals and weight gain of transplant group, no adhesions.....	17 gm.	2.418	24	>.02 <.05	2.492
Wt. gain of transplant animals with and without adhesions.....	10 gm.	2.213	19	>.02 <.05	2.539
Uterine wt. of ovariectomized animals and transplants without adhesions. . .	29 mg.	8.125	21	.01	2.831
Uterine wt. of transplants with and with- out adhesions.....	108 mg.	15.384	15	.01	2.947
Uterine wt. of transplants with adhesions and unoperated controls.....	98 mg.	2.793	30	.01	2.750

¹ Probability levels for significance of difference between various groups are also presented for direct comparison. ² Eight uteri available for this average. ³ Nine uteri available for this average.

RESULTS

The data obtained are given in table 1 and figure 1. The group carrying grafts in the spleen has been divided into those with and without adhesions. Inspection of figure 1 shows that the growth curve of the animals carrying transplanted ovaries in the spleen and showing no adhesions at autopsy, is qualitatively different from both the normal animals and the ovariectomized group. Since all four curves had essentially the same initial slope, the discrepancies observed cannot be ascribed to after effects due to surgery. (The weights of animals which died during the course of the experiment have not been included in the curves. Also culled out were any animals

in any group which showed a weight loss during the last 3 determinations. By these criteria animals were removed from all groups. It should be noted that such culling tended to reduce weight differences between the important groups, namely ovariectomized and experimental animals without adhesions.)

The growth curves presented indicate that ovarian secretions escaped the portal circulation and influenced the mode of growth of the animals. The *t*-test for significance of difference has been used to determine the validity of the observed differences

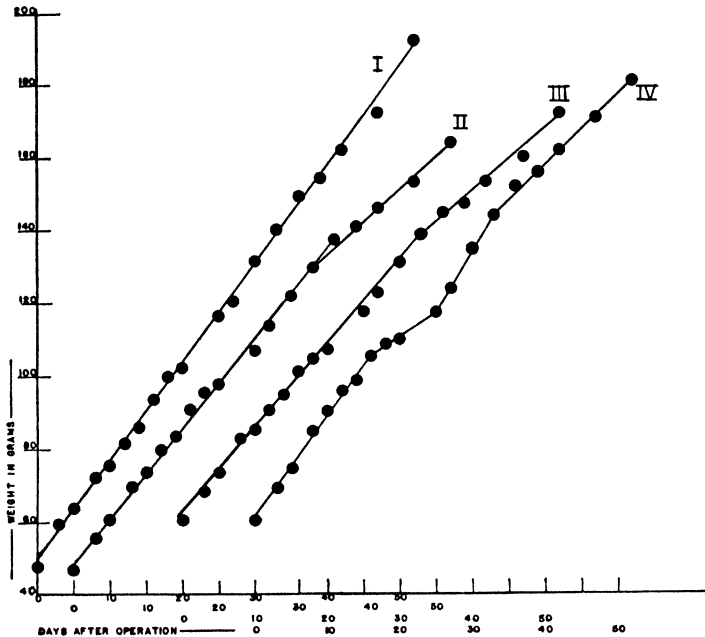


Fig. 1. GROWTH CURVES from 40 to 94 days of age of normal female rats, ovariectomized, and ovariectomized with autotransplantation of ovarian tissue into the spleen. All curves in figure have the weight axis in common. The day after operation axis has been set back over the scale in each instance to avoid confusing overlap. Day 0 for each curve begins at the first point. All animals were weighed on the same day. Thus, day 5 for the ovariectomized is day 5 for all others. Curve I = ovariectomized animals. Curve II = normal unoperated controls. Note overlapping break in this curve. It was so drawn to show that parallelism of upper portions of curves II and III may be more apparent than real. Curve III = ovariectomized and autotransplantation of ovaries into spleen. Adhesions present, collateral circulation bypassing portal system probably present in each instance. Curve IV = ovariectomized and autotransplantation into spleen, no adhesions, no bypassing collateral circulation.

in weight gain and uterine weight in the 4 groups of animals wherever such differences were questionable.

The calculations presented in table 1 show that: 1) The weight gain of the groups was: 139 grams for the ovariectomized, 122 for the adhesion-free experimental group, 112 for the experimental group with adhesions and 113 for the unoperated controls. The ovariectomized grew significantly faster than the adhesion-free group.

Those with adhesions grew at the same rate as the controls and significantly slower than the adhesion-free group. Thus the qualitative differences and similarities shown in the growth curves can be considered emphasized by the increments in weight achieved in a 54-day period. 2) The weights of the uteri of all 4 groups are significantly different from each other. The weights of the uteri of the adhesion-free grafted group are not as small as the ovariectomized, but they are smaller than those of the group with adhesions. The unoperated animals had uteri significantly larger than those of the group with adhesions to the splenic grafts. 3) The adrenal weights were uninfluenced by either ovariectomy or by splenic implantation of the ovaries.

The histological findings are in agreement with those of previous investigators, and also the weight data on the uteri. The ovarian tissue was almost completely luteinized. The amount of ovarian tissue ranged from twice to five times the volume of gonadal tissue seen in the unoperated controls.

The sections of the uteri showed endometrial growth in accord with the average weight of the group as it stood in the scale. The uteri of the ovariectomized animals presented the characteristic thin-layered picture. The experimental group showed uteri ranging from close to the ovariectomized type to the normal. The findings could not be better presented than on a weight basis.

At autopsy no positive vaginal smears were seen in the group without adhesions. Two such smears were found in the group with adhesions. Twelve were found in the unoperated controls. These data are too scant for elaboration.

DISCUSSION

Prior to considering the significance of our findings it should be reemphasized that a review of the literature has revealed to date no experiment in which ovarian transplantation to the spleen was made prior to puberty. In view of the absence of such data in the literature our results cannot be considered to controvert previous findings in experiments done with mature animals.

The data indicate that under the conditions of these experiments the liver is incapable of inactivating completely all the secretions of the ovary. That it can do so in good measure is shown by the data from the experimental group with adhesions to the spleen.

The differences in the growth curve, especially the peculiar changes in the rate of growth from the 15th to 35th day, shown by the adhesion-free group suggest also that a qualitatively different endocrine situation exists in these animals. If almost complete inactivation of ovarian secretion had occurred, a curve between the normal and the ovariectomized growth curve should seemingly have resulted.

SUMMARY

Data are presented on the growth rates over a 54-day period of immature 1) normal (23 animals) and 2) ovariectomized (16 animals) contrasted with similar data on animals of the same age receiving intrasplenic ovarian grafts and divided into 3) a group with no adhesions to the operated spleen (10 animals) and 4) a group with adhesions to the operated spleen (11 animals). The increased weight gain exhibited by the ovariectomized animals is not attained by animals of group 4, but the

weight gain of *group 3* animals is significantly greater than that of *groups 1* and *4*. Uterine weights of animals of *group 3* are significantly greater than those of *group 2* and significantly lower than those of *groups 1* and *4*. It is concluded that a complete inactivation of ovarian secretions effecting growth and uterine size was not attained after intrasplenic ovarian transplantation in immature rats.

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ACTOMYOSIN OF THE UTERUS¹

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INTEREST has been centered around the uterine muscle from the very beginning of biological and gynecological investigations. The response of the whole uterus or strips of uterine muscle to different agents has been investigated in different stages of the cycle and in pregnancy. Though these methods can be regarded as fairly correct from the physiologist's point of view, they do not yield much information about the functional units of the uterine muscle itself.

The function of the uterine muscle in labor is so profoundly different from that under ordinary conditions that the well-known increase of weight, cell-number, and size can hardly be considered solely responsible for this change. Even if we accept that the function of the uterine muscle is regulated by hormones, it seems very probable that the reason for the altered function lies in the changes that take place in the molecular structure of the muscle itself and in the quantitative and qualitative changes of the elementary contractile material.

In a series of experiments (1, 2) the author was able to show that this is actually the case. The contractile material of the uterine muscle shows a significant qualitative and quantitative change during the course of pregnancy.

These investigations were based upon the results of Szent-Györgyi and his collaborators (3) who have shown that the muscle protein actomyosin is responsible for muscular contraction. Actomyosin consists of two proteins, myosin, M, and actin, A. Both were isolated and purified. Neither of these two proteins is in itself contractile. If put together they form the AM complex.³ AM can be made into the form of a thread which 'contracts' *in vitro* in the presence of adenosine triphosphate, ATP, and ion constituents of the muscle fiber.

By applying somewhat modified methods of the Szent-Györgyi school to the study of the uterine muscle, the investigations discussed herein show that there also the contractile protein is AM. They show further that M and AM in the uterine muscle undergo significant changes during the course of pregnancy and that these are in good agreement with well-known facts of uterine physiology.

EXPERIMENTAL

Extraction of the Contractile Proteins. Szent-Györgyi's technique (3) for the extraction of AM was modified so as to extract all the AM present in the uterine muscle. About 2 to 4 gm. of material was put in a refrigerator at -14°C . immediately after removal from the body and kept at least 4 hours at this temperature. Then it was

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³ For convenience, myosin will hereafter be referred to by the initial M, actin by A, and adenosine triphosphatase by ATP.

cut into small pieces and 2 gm. of uterine muscle were ground for 10 minutes at room temperature in a mortar with 6 ml. 0.5M KCl, 5 mg. ATP and 2 gm. quartz sand. Centrifuging this pulp at 3000 rpm. for 15 minutes gave a clear fluid which was poured off and kept for 24 hours at 4°C. during which time the ATP was completely split. All the M and A are extracted by this method as further extraction with M, according to Straub (4), does not increase the yield.

Viscosimetric Measurements. The solution prepared as previously described was used for viscosimetric determinations. A simple Ostwald viscosimeter and a buffer consisting of 0.5M KCl and 0.1M K-veronal-acetate buffer pH 7, was used. The outflow time was determined first and then 0.1 ml. of a one per cent K-ATP solution was dropped directly into the viscosimeter, mixed by blowing, and the outflow time immediately redetermined. The solution was diluted with the above-mentioned buffer to get a relative viscosity (outflow-time of the solution/outflow-time of the buffer) of

TABLE 1. AMOUNTS OF M AND AM IN THE EXTRACT FROM ONE GM. MATERIAL

MATERIAL	M + AM	AM	M	PERCENT- AGE OF AM IN M + AM
	mg.	mg.	mg.	
Human cross-striated muscle.....	44	26	18	60
Human uterine muscle, nongravid.....	16	5	11	30
Human uterine muscle, in labor.....	22	13	9	60
Rabbit uterine muscle, non-gravid.....	13	6	7	45
Rabbit uterine muscle, in labor.....	17	12	5	70
Rat uterine muscle, nongravid.....	9	2	7	22
Rat uterine muscle, in labor.....	14	7	7	50
Cow uterine muscle, nongravid.....	10	3	7	30

1, 2 to 1, 4 in the presence of ATP. The data of the relative viscosities with and without the addition of ATP were used to calculate the amount of A, M, and AM present. Snellman and Erdös (unpublished) offer a simple method for this calculation. They are able to show with the help of the ultracentrifuge that A binds a definite amount of M to form AM. M or A in excess is left unbound in solution. They accept Straub's value of 1 A to 2.5 M for practical purposes and suggest an empirical curve, which enables one to read directly the values for A, M, and AM from the above-mentioned viscosity data.

Contraction of AM Threads. AM threads were prepared from the same solution which was used for viscosity measurement. The AM solution was extruded through a capillary into 0.05M KCl + 0.001 molar $MgCl_2$ where it solidifies in the form of a thread (3). Pieces of such AM threads 2 mm. long were suspended in the above-mentioned mixture, put under a microscope with a magnification of 18, and the length measured by an ocular micrometer. ATP was then added to the suspension fluid and the contraction of the thread was observed and timed.

RESULTS

The contractile proteins were extracted from human and animal uteri in different stages of pregnancy (nongravid, gravid, and in labor). Their amount was determined

by viscosimetry; threads were prepared and their contraction, under the action of ATP, was studied. $M + AM$ reach their highest value, in the investigated cases, in the human striated muscle. The AM represents about two thirds of the mixture (table 1).

The amount of $M + AM$ in nongravid human uterine muscle is about one-third of that in human striated muscle. (The AM is only one-fifth that in human striated muscle.) The AM in the nongravid human uterus is only one-third of the $AM + M$ mixture. Human uterine muscle in labor contains one half as much $M + AM$ as human striated muscle and the amount of AM is also one half, but the ratio $M:AM$

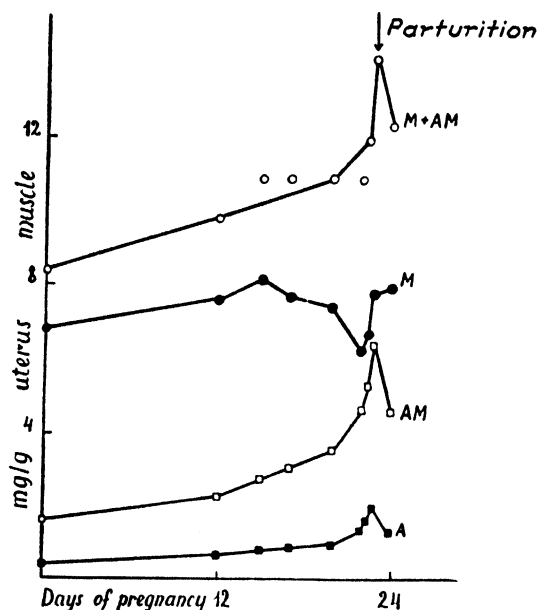


Fig. 1. AMOUNTS OF ACTIN, A, ACTOMYOSIN, AM, MYOSIN M, AND MYOSIN + ACTOMYOSIN, $M + AM$, in the uterus of the rat.

is equal to that of striated muscle. The amount of AM in the uterus during labor is two-thirds of the $AM + M$ value. Comparing the amount of $M + AM$ in one gm. of uterine muscle in the nongravid uterus and the uterus in labor we get 37 per cent higher value for the uterus in labor. In the former the AM is one-third of $M + AM$ but two-thirds in the latter.

We get lower values for both nongravid and parturient uteri of the rabbit compared with the human uterus, but the percentage of AM is higher than the corresponding values in the human uterus. The uterine muscle of the rat yields even lower values than that of human or rabbit, and even the percentage of AM is lower. The values for the cow seem to be the same as for the rat. Changes in the amount and ratio of the contractile proteins were studied in a series of rabbits and rats. The results are summarized in figure 1.

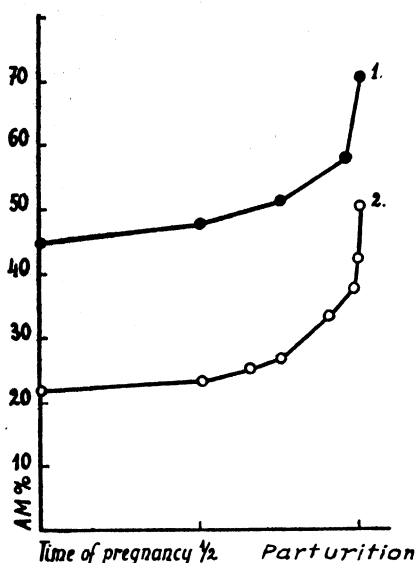


Fig. 2. PERCENTAGE OF AM IN AM + M from uterine muscle 1) of rabbit; 2) of rat.

increase in the first two thirds of the period of gestation, and suddenly in the last few days.

Our next question was, what effects can the alterations in the absolute amount of the M + AM and the ratio M:AM have on the contraction of our 'thread'? We have accepted that the thread can be regarded as a simplified muscle model. The characteristics of smooth muscle compared with striated muscle are usually summarized in the following 3 points: 1) longer latency period; 2) slower contraction; 3) smaller degree of contraction.

We paid much attention to these characteristics during the course of our investigation of the contraction of the threads. The period elapsing between putting ATA into the experimental solution containing the AM-tread and the beginning of the contraction, will be called the 'latency period'. The results are summarized in figure 3.

The threads prepared from the nongravid human uterus, which contains small quantities of M + AM and within this value a low percentage of AM, have a longer latency period, show slower progress and have a smaller degree of contraction than the threads prepared from human striated muscle. Threads prepared from the uterus in labor stand between striated and uterine muscle in thread-contraction characteristics.

The threads were always prepared from the solution of the first extraction. We could not prepare threads when the percentage of the AM in the AM + M mixture was below 30 per cent. These solutions could be easily brought to higher concentrations by precipitating the contractile proteins by dilution with subsequent centrifugation.

In the first two thirds of pregnancy A, M, and AM show a general slow increase. In the last third the values for M + AM do not change, but we can observe a very pronounced change in the M:AM ratio. At the beginning of labor the curves for M and AM nearly coincide. During labor the curves show a continued increase and they culminate at parturition. This is followed by a decrease in all values except that for M. The values for M are again slowly increasing. The A is decreasing and thus more and more M is getting into the 'unbound' state.

Changes in the amount of the contractile proteins can be clearly demonstrated by drawing a curve which represents the percentage of AM in the AM + M mixture. Figure 2 shows values for the rabbit and the rat. The slopes of the 2 curves are similar, but with higher values for the rabbit. The values slowly

gation and re-solution. Experience taught us to be careful with this method. Threads which readily contracted when prepared from the original solution more or less lost their ability to contract when prepared from precipitated and redissolved solutions. But the threads lost their contractility even when standing for some hours in the usual solution into which they were extruded ($0.05 \text{ KCl} + 0.001 \text{ MgCl}_2$). From the latter observations one may deduce that during the precipitation by dilution or during the immersion of the thread in this solution some kind of substance essential for the contraction of the thread is dissolved away. The dilutions in both cases seem to diminish the concentration of this substance to a value where it is ineffective.

That repeated washing with water changes the properties of myosin and that this change is due to the fact that some water-soluble material is removed from M by water was described by Szent-Györgyi (5). This material was isolated in an impure condition. By adding it to M the original properties (thread-contraction, enzymatic activity) could be restored.

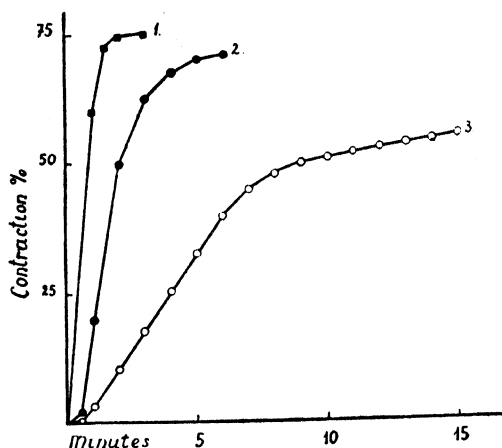


Fig. 3. PERCENTILE 'CONTRACTION' of AM threads plotted against time. 1) human cross-striated muscle; 2) human uterus in labor; 3) human nongravid uterus.

An aqueous extract was prepared by grinding one gm. uterine muscle with 3 ml. H_2O and sand. When this aqueous extract was added to a thread which had lost its contractility by precipitation or simply while standing, the contractility could be restored. The material present in the aqueous extract and responsible for this action has been tentatively designated 'X-factor.'

By adding X-factor to a thread prepared from nongravid human uterine muscle the contractility could be restored to a greater extent than when 'X-protein' itself was prepared from uterine muscle in labor and less contractility was obtained when X-factor was prepared from nongravid uterine muscle. We concluded that the nongravid uterus and the uterus in labor contain different amounts of X-protein.

Preparing X-factor (in one case) from a uterus which suffered spontaneous abortion in the third month of pregnancy, we found a higher concentration than in normal cases. Determining the amount of M + AM we got slightly higher values than usual 17 mg./gm. , and pronounced higher values for AM (50%).

One other case should be mentioned. The uterine muscle of an old primipara, whose labor did not progress for 48 hours, yielded 14 mg. of $M + AM$ /gm., which is even lower than nongravid uteri. The percentage of AM was as usual.

In the following discussion a working hypothesis is attempted to explain these experimental results.

DISCUSSION

These experiments suggest that uterine muscle considerably differs from striated muscle not only morphologically but even in its biochemical structure. Whether the substances taking part in contraction are identical in the two kinds of muscle cannot be stated at the present stage of the work. They are at least apparently identical, but they differ significantly in amounts and ratio of M and AM . The difference is so characteristic that it helps to explain even the differences in working capacity of the two kinds of muscles.

I consider the uterine muscle to be the best material for demonstrating the importance of the amount of $AM + M$ and of the $M:AM$ ratio for muscle function, the uterine muscle being the only tissue which undergoes a cycle of development under physiological conditions (during pregnancy). The uterine muscle in labor does more work than when nongravid, and must be considered more highly developed in that condition.

It is important to observe which substances are increased during this process and which are decreased after parturition.

Detailed investigations were made only of the changes in the amount of $M + AM$ and in the ratio $M:AM$. The problem of the X -factor was only touched upon and it suffices to state that this substance seems to increase during pregnancy as does the percentage of AM . The few pathological cases give only a hint. Abortion with its excessive contractility may possibly show us what is needed for contraction. On the other hand, the weak contractility in our second case might show what was lacking in this case and thus explain why this uterus was not working perfectly.

To summarize these results, we have found 3 factors which play important roles in the function of the uterine muscle. These are the amount of $M + AM$, the ratio $M:AM$, and the amount of X -factor. All 3 are low in nongravid uterine muscle compared with striated muscle; all 3 increase in pregnancy, reaching their climax during labor.

The uterine muscle seems to prepare itself to fulfill its physiological task by becoming similar in its contractile constituents to cross-striated muscle, and the values for uterine muscle in labor are really more like those of striated muscle than the nongravid uterine muscle. This development is very well demonstrated in the 'thread contraction'. The mechanism of this thread contraction is not yet clarified, and is a much discussed question, but the contractions of the uterine muscle threads show an astonishing consistency. Threads prepared from uterine muscles under the same condition always show contractions of similar character with a very small experimental error. Contraction of the threads increases with advancing pregnancy and reaches a maximum during labor. These facts make us accept the thread-contraction method as a complementary technic to the surviving-muscle experiments, the thread

contraction having, in fact, the great advantage that we know the amounts and ratios of the contractile substances present.

We suppose that the amounts and ratios of the contractile substances in the uterine muscle are regulated by hormones, but finally the function itself will depend partly on the contractile substances. Their role and importance in the function of uterine muscle cannot be doubted.

SUMMARY

The contractile proteins, myosin and actomyosin, were extracted from human striated muscle, human and animal uterine muscle, and the contraction of threads prepared from this material was studied. Two profound differences were found between striated and uterine muscle. Uterine muscle contains less $M + AM$ than striated muscle, and even the percentage of AM in the total sum of $M + AM$ is less. Threads prepared from nongravid uteri contract more slowly and to a smaller extent than threads from striated muscle.

The total sum of $AM + M$ and the percentage of AM increases during pregnancy in human and animal uteri. Both values reach their maxima during labor. Investigations carried out with rat and rabbit uteri show a significant increase of the AM percentage in the last period of gestation. The contraction of the threads increases with increasing values of the AM percentage, reaching the optimum during labor. In the rat the value for the sum of $M + AM$ decreases after parturition. The contraction of threads is profoundly influenced by a water-soluble factor, in absence of which they cease to contract.

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COMPARISON OF PROTEIN ANABOLIC PROPERTY OF VARIOUS ANDROGENS IN THE CASTRATED RAT^{1, 2}

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THE protein anabolic property of certain androgens has been established in the castrated dog and man (2). The studies described herein have been extended to the castrated rat.

PROCEDURE

The rats were castrated at approximately 4 months of age and kept in individual metal metabolism cages³ in an air-conditioned room maintained at 25.5 to 26.6°C. They were fed, weighed and injected at the same time each day. The diet was composed of casein 16.7, sucrose 61.2, hydrogenated vegetable oil 7.4, yeast (Fleischman's 2019) 9.2, Cellu flour 1.8 and Wesson's salt mixture 3.7, (3). Each batch of diet was analyzed for nitrogen, which averaged 2.95 per cent. A daily supplement of one drop of cod liver oil and one drop of a 34 per cent tocopherol concentrate from wheat germ oil⁴ diluted tenfold with Wesson oil. The rats were fed this diet at about 15 gm/day for about 2 months after castration, then the intake was gradually reduced to between 9 and 10 gm/day until constant body weight and nitrogen equilibrium were established and maintained. This procedure required 4 to 6 months.

The urine collections were made at 2-, 2- and 3-day intervals except in one experiment (fig. 1) when collections were made daily. Thymol and benzoic acid (4) were used as preservatives. The urine collection for each period was diluted to 500 ml. for analyses. The feces were collected daily and placed in 75 ml. of 30 per cent sulfuric acid in which they disintegrated on warming for about 10 minutes. The suspension was diluted to 500 ml. for analysis. The fecal periods of 7 days each were separated by adding 0.4 gm. of animal charcoal to the food of the last day of the period.

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² Many preliminary studies have been omitted. Some, however, have been reported at the Josiah Macy Jr. Conferences on Metabolic Aspects of Convalescence. Third meeting, 113, 1943; 7th meeting, 75, 1944; 12th meeting, 164, 1946. Parts of the data presented here have been included in a review (1) and in the Josiah Macy Jr. Conferences on the Metabolic Aspects of Convalescence, 16th meeting, 79, 1947.

³ These cages were purchased from Norwich Wire Works, Norwich, New York.

⁴ The tocopherol concentrate of wheat germ oil was generously provided by Distillation Products, Inc., through the courtesy of Dr. Philip L. Harris.

The nitrogen of the urine and feces was determined on duplicate—1 ml. aliquots of the diluted samples by the micro-Kjeldahl procedure. The diet was analyzed in triplicate by digesting approximately 1.5-gm. samples by the macro-Kjeldahl method, diluting to 500 ml. and distilling 10 ml. aliquots by the micro-Kjeldahl procedure.

Urea was determined by a modification of the urease-aeration technic (5); 1 ml. of urine was pipetted into a 1 x 8 in. test tube, to which was added 4 ml. of phosphate buffer and 0.5 ml. of urease suspension in phosphate buffer containing a Hynson, Westcott and Dunning urease tablet per 2 ml. The mixture was allowed to stand overnight at room temperature, then aerated into 10 ml. of a 2 per cent boric acid solution which was titrated with 0.0150 N HCl.

The testosterone propionate⁶ was supplied in sesame oil solution. The remaining androgens were dissolved in a small amount of acetone, the required amount of olive

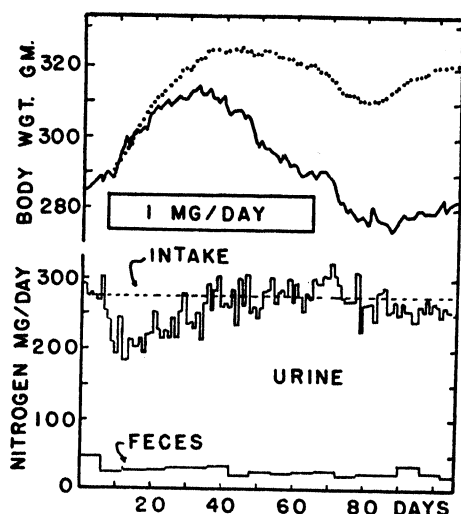


Fig. 1. EFFECT OF PROLONGED INJECTIONS of testosterone propionate on bodyweight and nitrogen excretion of castrated rats (Wistar). The observed body weights are graphed as a solid line. Body weight calculated from the nitrogen retained is shown by the dotted line. Urinary nitrogen is plotted above the fecal nitrogen. Daily dose is included in the block which indicates duration of the injections.

oil added and the acetone removed at 90 to 100° C. Injections were made subcutaneously usually in 0.1 ml. or less per day of solution. The pellets when used were prepared and implanted as previously described (6).

RESULTS

Characteristics of the Protein Anabolic Property of the Androgens. The effect of androgens on urinary nitrogen excretion and body weight (table 1) is illustrated by a representative experiment (fig. 1). Testosterone propionate at 1 mg. produced a sharp decrease in the urinary nitrogen excretion to a level which averaged 74 mg/day below that of the daily food intake. This new level of nitrogen excretion (maximum nitrogen retention per day) was maintained for several days and then the nitrogen excretion gradually returned to a level slightly below the pre-injection value, where

⁶ The testosterone propionate (perandren) and the other steroids were generously supplied by Ciba Pharmaceutical Products, Inc.

it was maintained for about 2 weeks (the 27th to 43rd day of injection) and then increased so that a slight negative nitrogen balance of about 10 mg/day was present and maintained. On cessation of injections, a small loss in nitrogen occurred for 8 days followed by a return to equilibrium and then a period of slight positive nitrogen balance.

The observed body weight (fig. 1) was sharply increased for about 16 days, then it gradually flattened and within 10 days began to decrease so that by about the 50th day of injection the body weight had returned to its preinjection value. On cessation of injections (after 58 days), a further decrease in body weight occurred followed

TABLE 1. PROTEIN ANABOLIC EFFECT OF VARIOUS STEROIDS IN THE CASTRATED RAT
(WISTAR, 280 to 320 GM. BODY WEIGHT)

STEROID	NO. OF RATS	DAILY DOSE	MAXIMUM		STEROID	NO. OF RATS	DAILY DOSE	MAXIMUM	
			Increased Body Wt.	Daily N Retained				Increased Body Wt.	Daily N Retained
		mg.	gm.	mg.			mg.	gm.	mg.
Testosterone propionate	4	0.125	12	35	Androstanol-17 α , one-3	1	1.0	7	25
	1	0.4	18	65		1	2.0	14	35
	3	1.0	18	60		3	3.0	17	55
(Pellet)	1	0.18	13	40	(3 pellets)	1	0.2	7	40
	1	0.4	8	10		1	2.0	4	10
	7	1.0	11	35	Androstanol-17 α , one-3, propionate 17	1	2.0	4	10
Testosterone	2	2.0	12	35		1	2.4	9	15
	3	3.0	16	65		3	2.0	12	35
	1	2 \times 2.0	23	80	Δ^4 -Androstenedione-3, 17	2	0.0	13	50
(1 Pellet)	1	0.3	18	60		1	4.0	11	35
	1	0.5	13	70		2	6.0	14	40
(2 Pellets)	1	0.4	6		Androstanedione-3, 17	1	2 \times 3.0	14	30
	1	1.0	6	30		1	0.63	6	20
	2	2.0	11	35	Androstanediol-3 α 17 α acetate-3	1	0.63	6	20
17-Methyltestosterone	3	3.0	15	40		1	2.0	8	20
	1	2 \times 2.0 (6)				1	2 \times 10.0	17	25
	1	2 \times 4.0 (28)	22	65	Androstanediol-3 α 17 α (9 pellets)	1	2 \times 15.0	15	20
						1	1.84	12	30
						1	1.84	12	30
					17-Methylandrostanediol-3 α 17 α				
					Androsterone acetate				
					Androsterone (9 pellets)				

Figures in parentheses represent days.

¹ These values represent the greatest average daily N retention produced by the steroid and are the averages obtained from the 3 lowest periods (7 days) of N excretion.

by a gradual increase. If the retained nitrogen is converted to protoplasm by the factor, 29.2, proposed by Albright (7), (dotted line, fig. 1) it immediately becomes apparent that the increase in observed body weight for about the first 10 to 14 days can be accounted for by the nitrogen retained but, thereafter, the observed body weight becomes increasingly smaller than that calculated from the nitrogen retained. Furthermore, although the observed body weight of the rat at the end of the experiment is less than that at the start, the protein content of the animal is greater. If, on the other hand, a 'small dose' (0.125 mg/day) of the androgen is administered for 21 days, then the observed and calculated changes in body weight are the same (table 1).

Results nearly identical to the first experiment (fig. 1) were obtained at a dose of

0.4 mg./day of the androgen (table 1). Furthermore, the changes in total urinary nitrogen were paralleled by changes in the urea nitrogen. In none of the experiments was there a significant change in fecal nitrogen output. (See fig. 1 and table 1.)

Steroids with Protein Anabolic Properties. These studies were carried out on rats of the Wistar strain from our colony. Since the duration of injections varied between 3 and 9 weeks within groups as well as for different doses and steroids, only the observed maximum increase in body weight and the maximum daily nitrogen are presented. Fecal nitrogen excretion was determined in all of the experiments except for testosterone propionate at 0.125 mg/day, but it was not affected by any of the steroids (see fig. 1). The average for all of the animals was 31 mg/day with a range of 26 to 36 mg. for individual rats.

All of the steroids except androsterone acetate produced a response in changes in body weight and nitrogen excretion similar to those illustrated for testosterone propionate (fig. 1). The androsterone acetate though administered in tremendous doses produced only a small retention of nitrogen. The increase in body weight was accounted for by the large amount of oil, 0.4 ml. and 0.6 ml/day, in which daily injections of the steroid had to be dissolved. The implantation of pellets of androsterone obviated this difficulty but the nitrogen retention was small as compared to similar experiments with other steroids (table 1).

Testosterone propionate proved to be the most effective of the steroids. It was effective at a dose as small as 0.125 mg/day and seemed to produce a maximal response at or below 0.4 mg/day (table 1). A pellet of testosterone propionate which was absorbed at the rate of 0.18 mg/day produced the same effect as the lowest injected dose of this androgen. Testosterone was not quite as effective as its ester until higher doses were used but when it was implanted as pellets (table 1), it proved to be just as effective as the esterified androgen. The smaller body weight response but maximal nitrogen retention in the double pellet experiment probably indicates that a high dose of the androgen was provided resulting in a greater concomitant loss of non-protein constituent (fat?) from the body.

It was found that 17-methyltestosterone gave roughly the same effects as testosterone. The last experiment with this steroid was of special interest. The animal was already in positive nitrogen balance and the injection of the androgen produced the expected increase in body weight but no appreciable effect on the urinary nitrogen excretion; therefore, after 6 days the already large dose was doubled with only a slight effect on the urinary nitrogen which soon increased and attained a value equal to that necessary for nitrogen equilibrium. The value for maximum nitrogen retained, therefore, was calculated from this and not the preinjection value. Similar observations have been made with other steroids. Thus, if an animal is already in strong positive nitrogen balance the effect of the androgen is to replace but not superimpose its effect on the existing nitrogen retention (8).

Androstanol-17 α , one-3 produced responses similar but probably slightly smaller than that produced by testosterone. The propionate of this compound in contrast to that of testosterone showed only a trace of activity at 2.0 mg/day. This was due to the extremely low solubility of this compound in tissue fluids (9).

Δ^4 -Androstenedione-3,17 and androstanedione-3,17 were effective but even at

doses as high as 6.0 mg/day did not produce effects comparable to the maximal responses produced by testosterone or testosterone propionate.

Androstanediol- $3\alpha,17\alpha$, acetate-3 and 17-methylandrostanediol- $3\alpha,17\alpha$ were studied at only one dose and only in one experiment each but they both demonstrated definite protein anabolic properties, as did androstanediol- $3\alpha,17\alpha$ at a very low dose absorbed from subcutaneously implanted pellets.

Simultaneous Comparison of the Protein-Anabolic Property of a Number of Steroids. Rats of the Holtzman strain were purchased and castrated at the same time. When they were adjusted to body weight and nitrogen equilibrium, they were divided into 6 groups of nearly equal weight. Then they were all injected for 21 days with

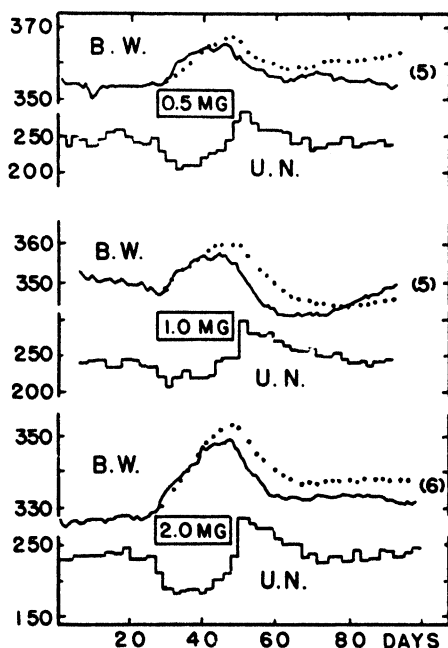


FIG. 2. EFFECT OF TESTOSTERONE on the urinary nitrogen excretion and body weight of castrated rats (Holtzman). Graphs represent average values of the number of rats indicated in parentheses. *B.W.* = body weight in grams; *U.N.* = urinary nitrogen in mg/day and the daily dose is placed in the block which indicates duration of the injections. Concentration of the steroid was 10 mg/ml. in olive oil.

the 6 different steroids at the same dose level. Each steroid except testosterone propionate and androstanediol- $3\alpha,17\alpha$ were studied at three different doses, 0.5, 1.0 and 2.0 mg/day. The order of the experiments was 2.0 mg., 0.5 mg. and 1.0 mg/dose. The intervals between the injection periods were 65 and 47 days. Testosterone propionate was not studied at the 1.0-mg. dose, and androstanediol- $3\alpha,17\alpha$ was not studied at the 2.0-mg. dose because of its low solubility in olive oil, 2 mg/ml. The data are summarized in table 2 and illustrated in detail by the experiments with testosterone (fig. 2).

Testosterone propionate was the most potent stimulator of protein anabolism. An increase of the dose from 0.5 to 2.0 mg/day produced a considerable further increase in body weight but only a slightly greater total nitrogen retention and a greater maximum daily nitrogen retention (table 2). On cessation of injections the

loss in body weight and nitrogen retention was much greater at the higher dose level. The observed body weight in both instances was much less than that calculated from the nitrogen retained.

Testosterone showed the same pattern of response (fig. 2) as its propionate but to a smaller degree until a dose of 2.0 mg. was employed when both produced

TABLE 2. COMPARISON OF PROTEIN ANABOLIC EFFECT OF SOME STEROIDS IN THE CASTRATED MALE RAT (HOLTZMAN, 330 TO 380 GM. BODY WEIGHT)

STERIOD ¹	NO. OF RATS	DOSE ² mg.	BODY WT. gm.	NITROGEN		BODY WEIGHT CHANGE			
				Prein- jection Urine mg/day	Max. Daily Reten- tion ³ mg.	Max. Incr.		Max. Loss	
						Obs'd	Calc.	Obs'd	Calc.
Testosterone Propionate	4	0.5	336	240	60	14	23	13	8
	6	2.0	338	238	71	20	24	23	20
Testosterone	5	0.5	354	245	35	12	14	11	9
	5	1.0	348	246	27	14	14	16	15
	6	2.0	328	238	50	21	26	16	16
Androstanol-17 α , one-3	4	0.5	353	241	20	6	6	6	7
	5	1.0	350	246	33	6	9	7	8
	5	2.0	352	241	51	14	19	9	6
Androstanediol-3 α , 17 α	5	0.5	357	244	21	5	7	2	5
	9	1.0	354	238	20	17	7	9	11
Δ^4 -Androstenedione-3, 17	5	0.5	348	239	8	4	3	3	9
	5	1.0	350	248	20	2	8	7	10
	6	2.0	346	240	35	12	14	7	11
Androstanedione-3, 17	5	0.5	338	238	11	6	4	2	0
	5	1.0	343	246	15	3	5	2	6
	5	2.0	342	244	23	11	9	3	4

Food intake varied among rats from 9.4 to 9.7 gm/day but the average for each group was 9.5 gm. or 280 mg. N/day.

¹ Androstanediol-3 α , 17 α was dissolved at 2 mg/ml. in olive oil and the rest of the steroids at 10 mg/ml.

² All of the steroids were studied simultaneously at the separate dose levels.

³ These values represent the greatest average daily N retention produced by the steroid and are the averages obtained from the 3 lowest periods (7 days) of N excretion.

⁴ The calculated body weight changes were obtained by multiplying the changes in N excretion by the factor 29.2 (7).

similar effects except that testosterone propionate was still more effective in the maximum nitrogen retained per day; 71 versus 50 mg/day (table 2).

Androstanol-17 α , one-3 was roughly 75 per cent as effective as testosterone (table 2). At the lowest dose, 0.5 mg., the calculated and observed body weights were identical during the injection period. At the 2 higher doses the steroid stimulated a greater calculated than observed body weight.

Androstanediol-3 α , 17 α at 0.5 mg. produced responses similar to those produced by androstanol-17 α , one-3; at 1.0 mg. it produced no further increase in nitrogen retention but a much greater increase in body weight (table 2). Thus, the observed body weight was greater than the calculated. Since the androstanediol-3 α , 17 α at

this dose required the injection of a daily volume of 0.5 ml. of oil solution, control animals were injected with this amount of oil for the same period of time. The body weight of these animals increased 11 gm., which accounts for the discrepancy between the observed and calculated body weights obtained with this steroid.

Δ^4 -Androstenedione-3,17 gave only small responses until a dose level of 2.0 mg. was used, at which it gave responses that were identical to those of testosterone at 0.5 mg. (table 2). The observed changes in body weight at the 0.5 mg. and especially at the 1.0-mg. doses unfortunately are somewhat masked by the small but constant rate of loss in body weight prior to the injections. Thus, the effect of the steroid on the body weight was partially nullified by this trend. A dose of 1.0 mg./day in identically maintained rats produced an increase of 10 gm. in observed body weight (unpublished). Such a phenomenon has been observed in a number of other instances with other steroids.

Androstanedione-3,17 was the least effective of all of the steroids compared. It, nevertheless, demonstrated definite protein anabolic properties (table 2) and the calculated and observed body weights closely equalled and paralleled each other.

DISCUSSION

The stimulation of nitrogen retention and increase in body weight induced in the castrated rat by androgens is similar to those observed in the dog and man (1). The 'wearing off' of these effects within a short period is probably due to the short life span of the rat. This same phenomenon probably would occur in the dog and man if the androgen injections were performed for a sufficiently long period. Indeed such an effect is evident in at least two studies (2).

The 'wearing off' effect of the androgens is not produced by an inhibitory effect of the administered androgen on the protein anabolic hormones of either the anterior pituitary (1, 10) or the adrenal cortex (1) for these same effects are produced in rats from which these organs have been removed. Furthermore, it is not due to the production of antihormones because the protein anabolic effect can be repeatedly demonstrated in the same rat.

In comparable studies Coffmann and Koch (11) demonstrated that the ability of castrated and normal rats to retain ingested creatine under the stimulation of 0.9 mg./day of testosterone propionate wore off in a period of time identical to that observed for nitrogen retention in our studies. These authors, moreover, noted phenomenal increases in body weight of their rats after 10 days of injection. Values were reported of 16 to 108 gm. with an average of 54 gm., or as much as 4 times that observed in our studies.⁶ Can it be that the ingested creatine is responsible for this phenomenal effect of the androgen? The experiments on testosterone propionate, therefore, were repeated but we were unable to find any change in the protein anabolic effect due to the addition of creatine to the diet.

No noteworthy differences are apparent in the effect of the androgens in the two strains of rats reported here or the Sherman-Carworth strain (unpublished).

The qualitative characteristics of the effect of the androgens on the body weight and urinary nitrogen are the same at the various doses and for most of the steroids.

⁶ Confirmed by personal communication with Dr. Coffman.

A rough quantitative comparison at various dosages of the different androgens is evident with respect to maximum increase in body weight, total nitrogen retained and the maximum nitrogen retained per day. On the basis of these criteria the relative protein anabolic potencies of the various steroids may be listed as follows: testosterone propionate > testosterone > 17-methyltestosterone > androstanol-17 α , one-3. > androstanediol-3 α 17 α > Δ^4 -androstanedione-3,17 > androstanedione-3,17 > androsterone acetate. The administration of testosterone, androstanediol-3 α 17 α , androstanol-17 α , one-3, and androsterone by subcutaneously implanted pellets greatly increased their efficacy.

It should be borne in mind that the distribution of the nitrogen retained to the various sites in the body differs markedly for the different steroids as indicated by renotrophic, androgenic (6, 12) and somatotrophic (9) studies in mice, and myotrophic and androgenic studies in guinea pigs (13). Furthermore, rats maintained in the same manner as those under metabolic study and injected for 21 days with 1 mg/day had seminal vesicles and prostates as follows: testosterone propionate, 4.48 gm.; androstanediol-3 α ,17 α , 3.51 gm.; androstanol-17 α , one-3, 3.04 gm.; and Δ^4 -androstanedione-3,17, 1.91 gm. (unpublished). The weight of these organs in normal rats was 2.48 gm. and in the castrated rats 0.21 gm. The kidneys of the treated rats also increased but in a different order from the accessory sex organs. The increases were androstanediol-3 α ,17 α , 0.94 gm.; androstanol-17 α , one-3, 0.50 gm. and testosterone propionate, 0.22 gm.; Δ^4 -androstanedione-3,17, 0.15 gm. Thus, the contribution of the organs to the increase in total body weight differs for the various steroids.

Although the ability of the androgens to stimulate nitrogen retention from dietary sources 'wears off,' this does not mean that stimulation of protein anabolic processes by the androgens has ceased. The accessory sex organs continue to grow (1, also unpublished data) at the expense of other tissues of the body. This reshuffling of the endogenous protein is probably responsible for the slight negative nitrogen balance observed on prolonged injection of the androgens.

The discrepancy between observed and calculated body weight occurs only when high physiological doses are used and is due at least in large part to an increased catabolism of endogenous carcass fat (11, also unpublished data).

SUMMARY

The injection of androgens into adult castrated rats in body weight and nitrogen equilibrium produces an immediate decrease in nitrogen excretion which is maintained at the new level (maximum nitrogen retained per day) for several days and then gradually returns toward the preinjection level. If the injections are further prolonged a slight negative nitrogen balance occurs. On cessation of injections, a negative nitrogen balance occurs for several days followed by a return to equilibrium and then a period of slight positive nitrogen balance. The changes in nitrogen excretion are paralleled by similar changes in urea excretion. The fecal nitrogen excretion is not altered.

The body weight is increased by the androgen for 10 to 14 days, then gradually ceases to increase and with further injections actually decreases. On cessation of

injections a further decrease in body weight followed by a gradual and small increase results. Calculation of the theoretical increase in body weight from the nitrogen retained indicates that the observed value at 'high' doses of steroid is not sufficient to account for the nitrogen retained, thus suggesting a concomitant loss in another constituent (fat?) of the body.

The maximum nitrogen retained per day, as well as the total nitrogen retained, and the maximum increase in observed body weight are related to the dose and efficacy of the steroids. Compounds at several dose levels have been compared with respect to these criteria and the following sequence of potency was noted: testosterone propionate > testosterone > 17-methyltestosterone > androstanol-17 α , one-3 > androstanediol-3 α 17 α > Δ^4 -androstenedione-3,17 > androstanedione-3,17 > androsterone acetate. In single experiments 17-methylandrostanediol-3 α 17 α and androstanediol-3 α 17 α acetate-3 demonstrated protein anabolic properties. When testosterone, androstanediol-3 α 17 α , androstanol-17 α one-3 and androsterone were implanted as pellets, their potency was greatly increased.

There was no noteworthy qualitative or quantitative difference between the Wistar and Holtzman strains of rats in responses to the steroids.

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COMPARISON OF THE PROTEIN ANABOLIC PROPERTY OF TESTOSTERONE PROPIONATE IN THE MALE AND FEMALE RAT¹

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THE nitrogen-retaining and body-weight increasing property of testosterone propionate and a number of other steroids in the castrated male rat has been described previously (1). These properties of testosterone propionate have now been studied in the female rat.

Rats were of the Wistar strain from our colony. Experimental procedures and the maintenance of the rats were as previously described (1). The testosterone propionate was provided at 10 mg/ml. in sesame oil.

RESULTS

Castrated Male Rats Compared with Normal Female Rats of Different Ages. The male rats were castrated at about 200 gm. body weight and were injected about 6 months later. The female rats were on constant food intake for approximately one month before the beginning of the experiment. The 4 series of experiments were carried out simultaneously.

The castrated male rats gave the expected responses (1) to the injection of testosterone propionate (fig. 1A, table 1). The female rats, on the other hand, were not only quantitatively (table 1) but also qualitatively (fig. 1 B, C, D) different in their responses which were further modified by the age (body weight) of the rats. The maximum daily nitrogen retention of the female rats was only one-fifth to one-third of that of the castrated male rats but was more prolonged. The total nitrogen retained, however, was not sufficient in any of the groups of female rats to account for the observed increase in body weight; the reverse of that obtained in the castrated male rats. On cessation of injections, the two heaviest groups had a loss in observed body weight less than the observed increase but only slightly greater than the calculated increase. The youngest group of female rats demonstrated only a very small decrease in observed body weight but a much greater decrease in the calculated body weight (table 1).

The increase in body weight of the female rats occurred at a much slower rate than that of the castrated male rats. Furthermore, while the body weight of the

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castrated male rats had ceased to increase after the 10th day and was actually decreasing after the 20th day, that of the female rats was still increasing at the termination of injections. In view of this difference between the two sexes, longer term experiments were carried out on 2 young adult female rats (fig. 2). The body weights

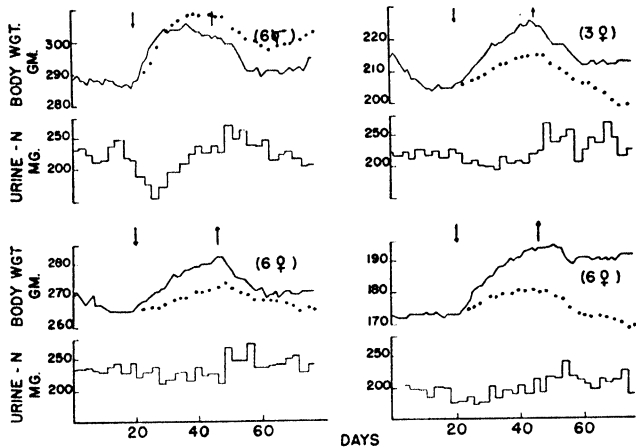


Fig. 1. COMPARISON of changes in body weight and urinary N excretion produced by testosterone propionate in castrated male rats with those in normal female rats of 3 different ages (body weights). The androgen was injected at 0.5 mg/day for 26 days. All of the experiments were carried out simultaneously. The dotted line represents the change in body weight obtained by multiplying the N retained with the factor 29.2 (2).

TABLE I. EFFECT OF TESTOSTERONE PROPIONATE (0.5 MG/DAY FOR 26 DAYS) ON URINARY N EXCRETION AND BODY WEIGHT OF CASTRATED MALE RATS AND NORMAL FEMALE RATS

RATS ¹	NO. OF RATS	BODY WT.	NITROGEN				BODY WEIGHT			
			Intake ²	Pre-injection urine	Max. ³ Retention	Max. Increase	Max. Loss		Obs'd	Calc. ⁴
							Obs'd	Calc. ⁴		
		gm.	mg.	mg.	mg/day	gm.	gm.	gm.	gm.	gm.
Castrated males	6	288	267	228	60	18	21	14	10	10
Normal females	6	265	275	235	12	15	8	10	12	12
	3	205	258	220	20	19	9	11	15	15
	6	173	242	199	19	20	9	4	12	12

¹ The rats in these experiments were studied simultaneously. ² The food contained 2.95 per cent N. ³ These values were obtained by averaging the N retained during the 3 periods with lowest N excretion. ⁴ These values were obtained by multiplying the N retained by the factor 29.2 (2).

did not stop increasing until after about 30 days of injections and demonstrated a small decrease only after another 30 days. On cessation of injections, no drop in body weight was evident in the rat injected for 37 days and only a small drop in the rat injected for 67 days. On reinjecting the first rat 25 days after ending the previous injections, a response similar to the initial one was obtained. However, after 14 days,

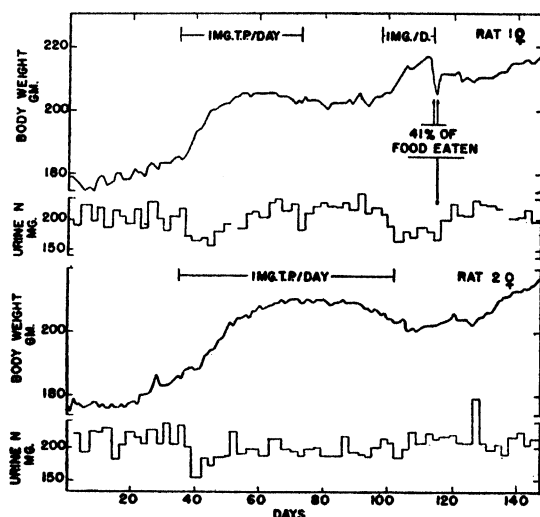


Fig. 2. EFFECT of prolonged (37 and 67 days) injections of testosterone propionate on the body weight and urinary N excretion of normal female rats. *Upper curve*, rat injected for 37 days. *Lower curve*, rat injected for 67 days.

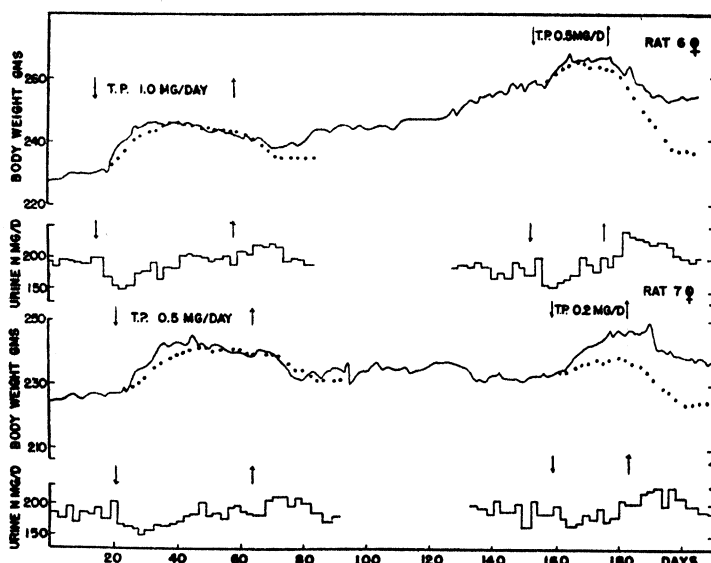


Fig. 3. EFFECT of testosterone propionate on body weight and urinary N excretion of ovariectomized rats. Dotted line represents the change in body weight obtained by multiplying the N retained with the factor 29.2 (2).

the rat ate only 41 per cent of its allotted food intake; therefore the injections were stopped. In these experiments, too, the increase in body weight exceeded by far the amount that could be accounted for by the nitrogen retained.

Ovariectomized Rats. The rats were operated on 40 days before the first experiment (fig. 3). Responses in body weight and nitrogen excretion were qualitatively

and quantitatively (table 2, fig. 3) intermediate between those observed in the castrated male and the normal female rats (table 1, fig. 1).

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE ON URINARY NITROGEN EXCRETION AND BODY WEIGHT OF THE OVARECTOMIZED RAT (WISTAR)

NO. OF RATS	DOSE	DAYS	BODY WT.	NITROGEN		BODY WEIGHT			
				Preinjection ¹ urine	Retention ²	Max. Incr.		Max. Loss	
						Obs'd	Calc. ³	Obs'd	Calc. ³
	mg/day		gm.	mg/day	mg/day	gm.	gm.	gm.	gm.
7	0.2	25	233	187	18	13	5	9	16
6	0.5	25	258	184	26	8	6	29	26
7	0.5	43	227	185	28	16	14	10	10
6	1.0	43	230	194	42	16	16	14	11

¹ The N intake for each rat was 233 mg/day. ² These values were obtained by averaging the N retained during the 3 periods with lowest N excretion. ³ These values were obtained by multiplying the N retained by the factor 29.2 (2).

DISCUSSION

The presence of the ovaries in the rat as well as in the woman (3) decreases the ability of testosterone propionate to stimulate nitrogen retention. The marked discrepancy between the observed increase in body weight and that calculated from the nitrogen retained cannot be explained at this time. Part of the resistance of the female rat to the nitrogen-retaining property of testosterone propionate apparently is provided by the ovaries for the removal of these organs made the rats intermediate between the castrated males and the normal females in their responses to the androgen.

It is of interest that hypophysectomized male rats also have to be injected for a much longer period of time than castrated male rats before a drop from the maximum increase in body weight is observed (4).

SUMMARY

Testosterone propionate produces a much smaller nitrogen retention in the normal female rat than the castrated male rat. Furthermore, the increase in body weight produced in the female rat is much greater than can be accounted for by the nitrogen retained and the maximum increase is obtained at a slower rate but is maintained for a longer period of time than in the castrated male rat. The ovariectomized rat responded to the androgen in a manner intermediate between the castrated male and the normal female rat.

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COMPARISON OF PROTEIN ANABOLIC PROPERTIES OF TESTOSTERONE PROPIONATE AND GROWTH HORMONE IN THE RAT^{1,2}

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ANDROGENS (1) and the growth hormone of the anterior pituitary (2) are potent stimulators of protein anabolism. The present report is concerned with a comparison of the effect of these hormones on total nitrogen balance and body weight.

PROCEDURE

Experimental methods were as previously described (3).

All the rats were autopsied at the end of the experiments. The pituitary site of the hypophysectomized rats was carefully examined both grossly and histologically, and the thyroid, adrenals, testes and seminal vesicles and prostates were weighed and studied histologically. The hypophysectomized rats showed the expected changes.

The testosterone propionate³ was provided as an oil solution containing 10 mg/ml. The growth hormone extract was prepared from beef pituitary. It contained "1.1 U/mg. in the 6-month-old plateaued female rat growth test"⁴ and "in the hypophysectomized immature female rat,⁵ 0.1 mg/day for 10 days produced an average increase of 14.1 gm. in body weight. It showed no corticotrophin activity but at a total dose of 2.5 mg. it repaired the interstitial cells of the ovary and the thyroid of the hypophysectomized rat." The extract was supplied as a dry powder and was dissolved as follows: 100 mg. was placed in a 15-ml. centrifuge tube set in an ice-water bath, 8 ml. of ice-cold N/100 sodium hydroxide was added, and the suspension

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² Parts of these data have been reported before the American Physiological Society, *Federation Proc.* 6: 144, 1947; *Josiah Macy Jr. Foundation Conferences on Metabolic Aspects of Convalescence*, 14th meeting, 169, 1946 and 16th meeting, 79, 1947 and in a review article in *Conference on Steroid Hormones*, Madison, Wisconsin, 1948. In press.

³ The testosterone propionate (perandren) was provided by Ciba Pharmaceutical Products, Inc., through the courtesy of Dr. Ernst Oppenheimer.

⁴ The growth hormone powder, Rx 70832, was provided and assayed by Parke, Davis and Co. through the courtesy of Drs. D. A. McGinty and L. W. Donaldson.

⁵ These assays were performed by the Institute of Experimental Biology, Berkeley, California, through the courtesy of Dr. C. H. Li.

vigorously stirred at frequent intervals for 30 to 60 minutes. Then the mixture was titrated to pH 9 (Universal indicator, Eastman Kodak Co.) with N/50 phosphoric acid, made to 10 ml. with distilled water, stirred and centrifuged. The supernatant solution was decanted into rubber-capped vials and kept at 0° to 5°C. A maximum of one week's supply was prepared at one time. All injections were made subcutaneously.

RESULTS

Castration. Food intake of rats of the Wistar strain from our colony was adjusted over a 3-month period until the body weight and nitrogen excretion were constant. Castration produced a slight increase in the urinary nitrogen excretion during the

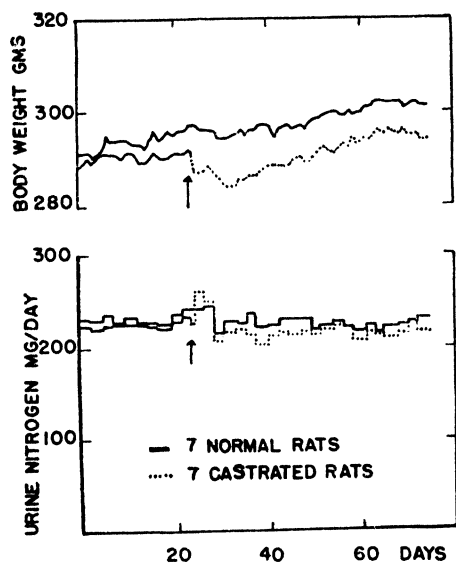


FIG. 1. EFFECT OF CASTRATION ON nitrogen excretion and body weight of male rats.

first 4 days followed by a drop to a level slightly below and then a gradual return to the preoperative value (fig. 1).

The initial decrease in body weight (fig. 1) was equal to the weight of the removed testes. A further small drop in body weight, however, occurred during the 4th to 6th post-castration days followed by a gradual increase. After about 40 days the difference in body weight between the non-operated and operated rats was equal to that at the time of castration.

Hypophysectomy. Adult male rats of the Sprague-Dawley strain were hypophysectomized by the Hormone Assay Laboratories (Chicago) and shipped with unoperated controls by plane. They were immediately placed on a fixed food intake and the studies begun.

The urinary nitrogen excretion of the normal rats was constant and the balance slightly positive (fig. 2). The difference between intake and output of nitrogen was not as great as indicated on the graph because the fecal nitrogen, which would have been about 30 mg/day (3), was not determined. Hypophysectomy produced a very marked negative nitrogen balance which gradually lessened but was evident even after eighteen days, for the urinary nitrogen output alone was equivalent to the food intake.

Body weight of the normal rats decreased an average of 12 gm. during the first 2 days, remained constant until about the 20th day then began gradually to increase even though the nitrogen excretion remained constant, an apparent caloric readjustment which is the usual observation in this laboratory. Body weight of the hypophysectomized rats, on the other hand, decreased rapidly and continuously for the 18 days

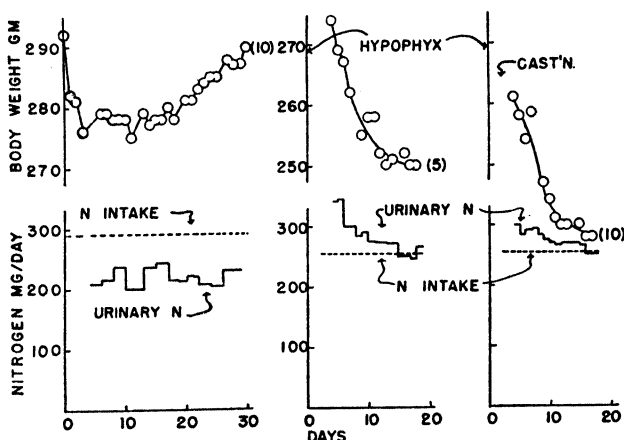


FIG. 2. EFFECT OF HYPOPHYSECTOMY on body weight and nitrogen excretion of male rats. Data for the normal controls are presented in the graph on the left.

studied. Furthermore, the loss in body weight was about twice that observed in the normal rats (fig. 2). Castration of 10 of the rats on the 3rd day after hypophysectomy (fig. 2) did not alter the effects of the loss of the pituitary.

Protein Anabolic Effect of Testosterone Propionate and Growth Hormone in the Castrated Adult Rat. Rats of the Wistar strain from our colony were castrated at about 225 gm. body weight and these experiments were carried out 6 to 8 months later. Food intake was 9.5 gm/day.

Growth hormone and testosterone propionate produced similar maximal decreases in nitrogen excretion (fig. 3) but the action of the growth hormone occurred sooner. After several days the nitrogen retention decreased but to a greater degree in the case of the androgen than the growth hormone. On cessation of injections there was a marked loss in nitrogen which reached the same degree for both hormones. After several days the nitrogen excretion returned to normal levels.

The 2 hormones produced identical and parallel increases in body weight of the rats for 14 days, except that the effect of the growth hormone was evident one day sooner than that of the androgen. Then while the growth-hormone-treated rats continued to increase in weight, the androgen-treated rats stopped and later actually began to lose weight. On cessation of injections both groups of rats showed a sharp decrease in body weight followed by a period of maintenance at the lower level and finally a slight increase.

In a single experiment with a much greater dose of growth hormone the effects were somewhat different. The nitrogen excretion decreased sharply and then returned to approximately the preinjection level. On cessation of injections, a very marked

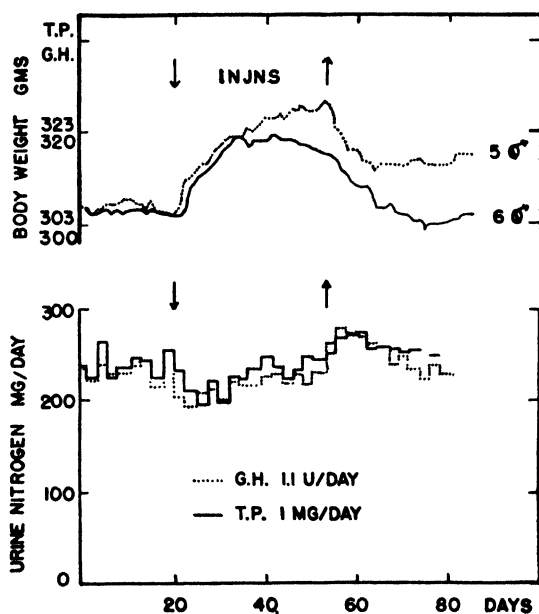


FIG. 3. COMPARISON OF THE EFFECT OF testosterone propionate and growth hormone on nitrogen excretion of castrated rats.

negative nitrogen balance occurred. The body weight increased sharply for about a week, stopped increasing and with further injections began to decrease, an effect similar to that obtained with the androgen. On cessation of injections, there was a precipitous loss in body weight to about 35 gm. below the preinjection level.

Protein Anabolic Effect of Testosterone Propionate and Growth Hormone in the Hypophysectomized Rat. Rats of the Sprague-Dawley strain were hypophysectomized by Dr. D. J. Ingle⁶ of the Upjohn Company and shipped by plane on the next day. As was expected the rats ate less and less food but finally came to body weight and

⁶ The author is grateful for this very generous cooperation.

nitrogen equilibrium at a food intake which varied from rat to rat between 5 and 7 gm/day. Because experiments were carried out for different periods, only representative graphs are given (figs. 4, 5) and the rest of the experiments are presented in

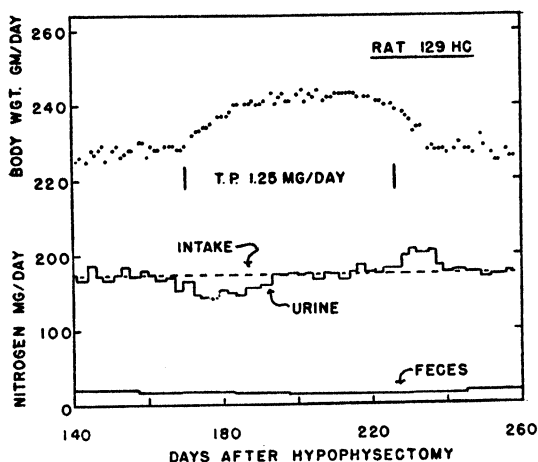


FIG. 4. EFFECT OF TESTOSTERONE PROPIONATE on body weight and nitrogen excretion of the hypophysectomized-castrated male rat.

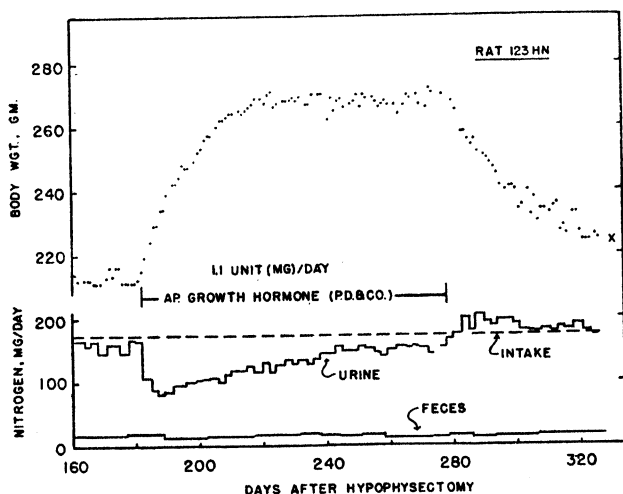


FIG. 5. EFFECT OF GROWTH HORMONE on body weight and nitrogen excretion of the hypophysectomized male rat.

tables 1 and 2 indicating only maximum increases in body weight and maximum daily nitrogen retained.

Both hormones stimulated nitrogen retention which gradually decreased on

continuation of the injections (figs. 4, 5). The effect of the growth hormone (fig. 5, table 2) not only was greater but also more prolonged than that of the androgen (fig. 4, table 1). Furthermore the androgen was less and the growth hormone more effective

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON BODY WEIGHT AND NITROGEN EXCRETION OF HYPOPHYSECTOMIZED ADULT MALE RATS

RAT NO. ¹	INJECTIONS		MAX. BODY WT. INCREASE	MAX. N RETENTION	REMARKS
	Dosage	After Hypophy.			
	mg/day	days	gm.	mg/day	
121 HC	1.0	34-56	8	20	Intake irregular, 72-105th day.
122 HC	1.0	63-109	12	25	
120 HN	1.0	60-110	10	15	
126 HN	1.0	63-101	9	15	
Averages			10	19	
122 HC	1.25	170-185	10	25	Intake irregular, 175-210th day.
129 HC	1.25	170-226	15	30	
131 HC	1.25	170-233	11	25	
125 HN	1.25	169-204	7	25	
Averages			11	26	
129 HC	2.0	63-101	13		Intake irregular, 88-99th day.
128 HN	2.0	63-109	18	20	
130 HN	2.0	60-101	9	20	
Averages			13	20	
131 HC	5.0	78-108	8	35	Intake irregular, 89-112th day.
132 HC	5.0	63-109	17	20	Intake irregular, 74-78th day.
134 HN	5.0	63-101	11	30	
Averages			12	28	

¹ HC = hypophysectomized-castrated; HN = hypophysectomized.

TABLE 2. EFFECT OF ANTERIOR PITUITARY GROWTH HORMONE ON BODY WEIGHT AND NITROGEN EXCRETION OF HYPOPHYSECTOMIZED ADULT MALE RATS

RAT NO. ¹	INJECTIONS		MAX. INCREASE IN BODY WGT.	MAX. N RETENTION
	Days after hypophysectomy	Units		
			gm.	mg/day
134 HN	170-260	0.55	47	60
120 HN	163-238	1.1	76	60
123 HN	162-258	1.1	58	75
126 HN	128-257	1.1	38	70
121 HC	170-260	1.1	72	70
Averages			61	69

¹ HN = hypophysectomized. HC = hypophysectomized and castrated.

in the hypophysectomized than in the castrated rat (fig. 3). On cessation of injections there was a loss of nitrogen for several days before equilibrium was reached again. The fecal nitrogen excretion was not affected by either of the hormones (figs. 4, 5).

The growth hormone produced a much greater increase in body weight (fig. 5, table 2) than the androgen (fig. 4, table 1). Furthermore, the increase in body weight was maintained as long as the growth hormone was injected, i.e., 90 to 129 days (table 2). The maximum level attained in the androgen-treated rats (fig. 4) was maintained for a much longer period of time than in the castrated rats (fig. 3) but eventually, after 50 days, began to decrease (fig. 4). On cessation of injections, a sharp loss in body weight occurred in both groups of rats followed by maintenance at the lower level (figs. 4, 5).

The androgen was administered at several dose levels (table 1) but no difference in the responses could be detected. Several of the rats, however, after about 7 days of injections showed a decrease in appetite which varied in duration for the different rats.

The subcutaneous implantation of a 14 to 15-mg. pellet of testosterone which provided a daily dose of 0.33 mg., produced an effect on body weight and nitrogen retention similar to those produced by injection of testosterone propionate.

DISCUSSION

The changes in body weight and nitrogen excretion after castration are identical to those observed after cessation of injections of androgens (3) and are similar to those observed after castration of the adult rat eating *ad libitum* (4). The rats on constant food intake, however, show a small extra excretion of nitrogen during the first 4 days which may be accounted for by the atrophy of the accessory sex organs. This small loss of nitrogen may have been present in the rats eating *ad libitum* but was not detected because of the conditions of the experiment (4). The very marked excretion of nitrogen after hypophysectomy is in agreement with that observed in male and female rats eating *ad libitum* (5) but not in female rats on a constant food intake (6). The latter were reported to be in nitrogen equilibrium. In any event, removal of the pituitary in the male rat has a much more profound effect on nitrogen metabolism than the removal of the testes. In both instances, however, the animals readjust their metabolic processes to a state of equilibrium.

The ability of growth hormone to decrease the urinary nitrogen excretion has been amply demonstrated (2). Unfortunately, in all of these experiments the period of injection was extremely brief; therefore, the phenomenon of gradual disappearance of the nitrogen-retaining property of this hormone was not observed except in a very recent report (7). It is of interest that although the rats return toward nitrogen equilibrium, they do not go into negative nitrogen balance until the injection of the growth hormone is stopped. Therefore, since both these phenomena, the 'wearing off' and the rebound effects, occur in the hypophysectomized as well as the non-hypophysectomized rat, they cannot be due to a suppression of the endogenous production of growth hormone.

The ability of androgens to produce nitrogen retention and to increase the body weight of the hypophysectomized male rat plus similar observations in the hypophysectomized female rat (6), the hypophysectomized-castrated dog and in patients with hypopituitary activity (1) provide conclusive proof that the protein anabolic property of the androgens is not mediated through the pituitary. Furthermore, the

sites (8) and mechanism (9) of action of these two hormones are different. The absence of the pituitary, however, does modify the effect of androgens on both body weight and nitrogen excretion. The maximum increase in body weight produced by the androgen persisted for a much longer period of time in the hypophysectomized than in the castrated rat and the amount of nitrogen retained was less. These modifications may be due to the lower metabolic status of the hypophysectomized rat. Furthermore, occurrence of a nitrogen loss or rebound on cessation of injections cannot be due to a suppression of the production of endogenous growth hormone. Since both the 'wearing off' effect and nitrogen rebound occur also in normal (1), castrated (3, 10) and adrenalectomized-castrated (10) rats injected with androgens, it would seem that these phenomena are more likely due to a hormone-tissue relationship than the inhibition of the endocrine function of another gland.

The greater effectiveness of the growth hormone in the hypophysectomized rat than in the castrated rat and the reverse in the case of the androgen emphasizes the importance of the metabolic status of the animal in its ability to respond to the stimulus of a hormone.

The decrease in appetite produced by testosterone propionate in the hypophysectomized rats was not as great or as harmful as that observed in hypertensive rats (11). The failure to observe this effect on appetite in female hypophysectomized rats (6) is probably due to the very short duration of this experiment.

SUMMARY

Castration of adult male rats in body weight and nitrogen equilibrium produced a slight increase in nitrogen excretion and a small drop in body weight during the first few days followed by a period of slight nitrogen retention and gain in body weight. Hypophysectomy, on the other hand, produced a rapid and large loss in body weight accompanied by a very marked extra excretion of nitrogen, which gradually returned to equilibrium. The injection of testosterone propionate at 1 mg/day and growth hormone at 1.1 units/day into castrated male rats on constant food intake produced identical increases in body weight and nitrogen retention for 14 days, then the effect of the androgen on body weight ceased to persist while that of the growth hormone continued. Also, the nitrogen excretion of both groups of rats returned toward the preinjection level. On cessation of injections a rebound or loss of nitrogen occurred in both groups of rats.

Testosterone propionate at doses of 1 to 5.0 mg/day and a pellet of testosterone (0.33 mg/day) produced in the hypophysectomized male rat similar increases in body weight and nitrogen retention. The effect on body weight persisted for a much longer period than in the castrated rat while the nitrogen excretion decreased to a much lesser degree but as in the case of the castrated rat gradually returned to equilibrium on continuation of injections and increased for several days on cessation of injections. Growth hormone produced a much greater effect on nitrogen retention and increase in body weight in the hypophysectomized than in the castrated rat and also was more effective than the androgen. The nitrogen-retaining property of this hormone also did not persist but gradually returned to normal. The increase in body weight, however, was maintained at the 'plateaued' maximum for the duration of

the injections. On cessation of injections a sharp drop in body weight and an increase in nitrogen excretion occurred.

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LEUCOCYTE PICTURE OF THE RAT: RELATION OF ADRENAL AND SPLEEN

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CERTAIN dyscrasias of the blood have been shown to be related to a disturbance in the function of the spleen (1, 2), or in the function of the adrenal cortex (3-5) in clinical and experimental subjects. Data in the literature indicate that some of these changes are of a similar pattern when the affecting agent is the adrenal or the spleen.

There is general agreement that a marked leucocytosis appears after spleenectomy (2) and after adrenalectomy (4). Spleenomegaly, which is believed to be a sign of hyperactivity of this organ, is associated with a neutropenia in these cases (2, 6), whereas a hyperactivity of the adrenal cortex in clinical cases (5, 7) and in animal experiments (8-11) has been associated with a neutrophilia among other changes in the white blood cell composition. There are reports of the induction of a leucocytosis, which was primarily a neutrophilia, following the subcutaneous injection of adrenaline in patients and animals before and after spleenectomy, and in spleenomegalic patients (12-16). Adrenaline injections have also been used to stimulate the pituitary-adrenal cortex axis in normal animals and in patients with a resulting leucocytosis and neutrophilia (7, 17-19). A significant negative correlation has been reported (19) between the adrenal ascorbic acid concentration and the absolute number of circulating neutrophils in the normal rat. After the injection of adrenaline the adrenal ascorbic acid was depressed and was no longer correlated with the neutrophilia which was present. Since the spleen has a fairly high concentration of ascorbic acid and is responsible to adrenaline, these lines of evidence suggested that the spleen's role in the leucocytic regulation might be reflected in changes in its ascorbic acid content or influence on the ascorbic content of the adrenal glands.

This paper presents evidence that the neutrophilia observed in the spleenectomized rat is not associated with a depletion of the adrenal ascorbic acid as in the case of neutrophilia which follows injection of adrenaline. The data show that the spleen is involved in the neutrophilia and the lymphopenia which follows 3 hours after the injection of adrenaline in rats.

METHODS

Adult male rats of the Sprague-Dawley strain weighing 200 to 250 gm. were used in these studies. Rats were given Purina Chow and tap water (the drinking solution of the adrenalectomized rats was a 1% NaCl solution) and greens at least once a week. Total and differential leucocyte counts were made by the direct method of Randolph (20) on tail blood. The ascorbic acid content of the adrenal glands and spleen was determined by the method of Roe and Keuther (21). The adrenal glands were

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removed and cleaned of extraneous tissue and weighed on a Roller-Smith balance to the nearest tenth of a milligram. A mid-section of spleen (estimated to weigh about 100 mg.) was removed and blotted on filter paper and weighed on the precision balance to the nearest tenth of a milligram. Most sections weighed between 90 and 150 mg. Evipal anesthesia administered intraperitoneally was used in all surgical procedures and when tail blood was drawn.

The differential blood picture of the several groups of rats used in this study are arranged in two general categories: 1) Before surgery, and 6 or 7 days after surgery; 2) 8 or 9 days after surgery immediately before and 3 hours after the last of a series of adrenaline injections. The adrenaline was a 0.02 per cent solution in physiological saline which was injected subcutaneously at a dose level of 0.02 mg/100 gm. of body weight at 3-hour intervals. The groups of rats in *category I* were: 13 adrenalectomized; 6 spleenectomized; 5 adrenalectomized-spleenectomized; 6 sham-spleenectomized; and 4 sham adrenalectomized-spleenectomized rats. The groups of rats in *category II* were: 12 adrenalectomized; 6 spleenectomized; 5 adrenalectomized-spleenectomized; and 4 sham adrenalectomized-spleenectomized rats. A group of 6 normal intact rats before and after adrenaline injections was also included.

The significance (value of *P*) of the Mean Difference was computed from the before and after values of the total white blood cells, absolute number of circulating lymphocytes, polymorphonuclear leucocytes, and eosinophiles per cubic millimeter of blood in each of the groups of rats. The relation of the circulating number of polymorphonuclear leucocytes per cu. mm. of blood to the ascorbic acid concentration of the adrenals and the spleen of each rat in the following groups is represented: normal intact, 14 rats; 3 hours after the last adrenaline injection, 8 rats; 3 hours after 0.85 per cent saline injections, 6 rats. The relationship of the adrenal ascorbic acid concentration is also represented for 7 spleenectomized and 8 sham-spleenectomized rats. The significance of the Difference between the Means of the ascorbic acid concentration of the adrenal glands, and of the spleens was computed for the several groups of rats.

The time lapse of 3 hours after the last adrenaline injection was chosen as an end-point for the demonstration of the polymorpho-leucocytosis and its relation to the concentration of the adrenal ascorbic acid on the basis of data in the literature. It has been shown that the induced polymorpho-leucocytosis and the depression of the adrenal ascorbic acid concentration are at a maximum approximately 3 hours after adrenal cortical stimulation (7-9, 18).

The role of the spleen in natural and acquired resistance to infection has been reviewed by Perla and Marmorston (22). Rats with latent infections of *Bartonella muris* rapidly succumb to spleenectomy. Some strains of rat are not infected with *Bartonella*. A few of our spleenectomized rats were kept alive in our colony for several months without any special attention.

RESULTS

The mean absolute values of the total number of white cells, lymphocytes, polymorphonuclear leucocytes (in the rat these are practically all neutrophiles), and eosinophiles per cu. mm. of blood are shown in figure 1. Tests for statistical signifi-

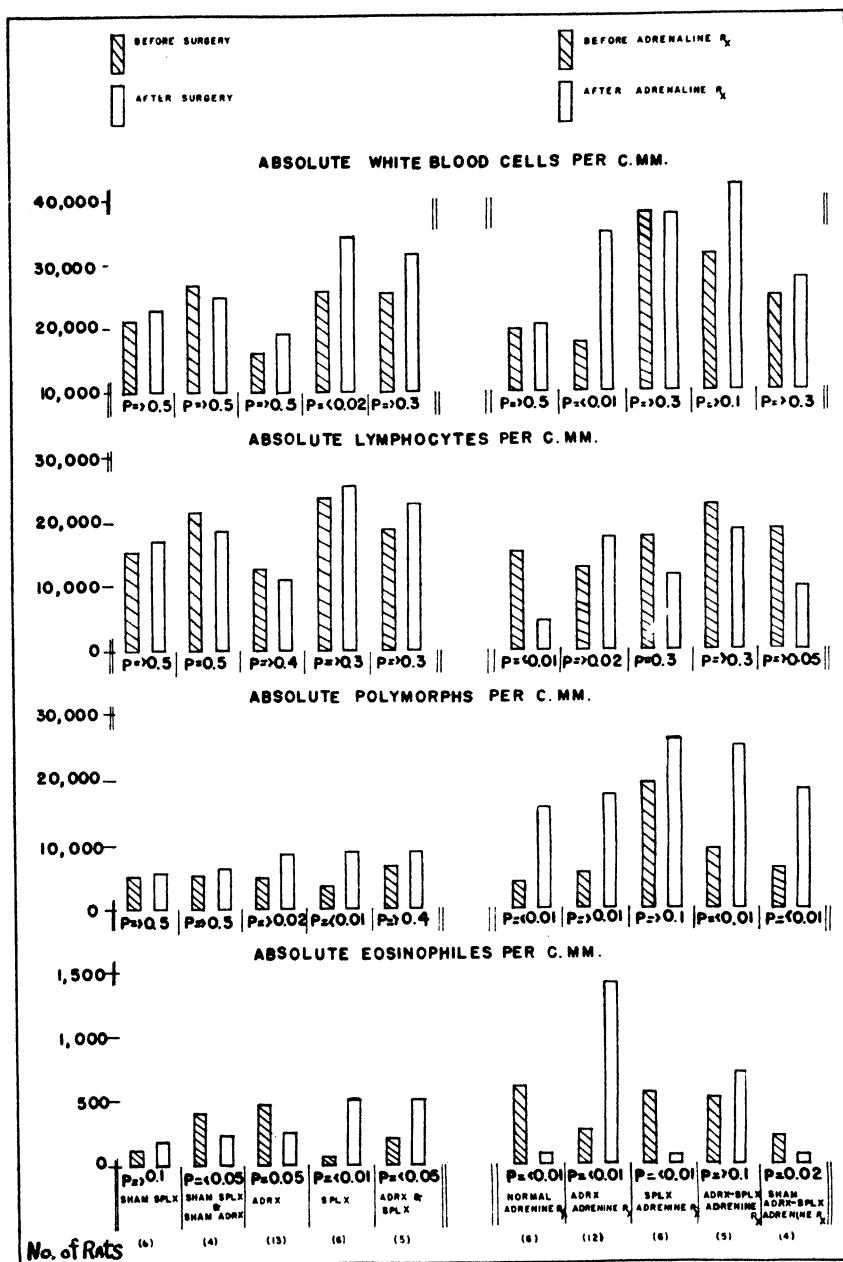


FIG. 1. MEAN VALUES AND SIGNIFICANCE (value of P) of the Mean Difference before and after the experimental procedures indicated on the leucocytic picture of different groups of rats.

cance for the Mean Difference of the white blood cell components in each group of rats before and after the different procedures resulted in the following significant differences of mean values.

Total White Blood Cells per cu. mm. of Blood. A significant increase of total white blood cells was observed after splenectomy, and in the adrenalectomized group 3 hours after the adrenaline injections.

Lymphocytes per cu. mm. of Blood. No statistically significant change occurred in the mean lymphocyte values following the operative procedures in any of the groups of rats. Significant depression of the mean lymphocyte values was found in the normal and sham-operated groups of rats after the adrenaline injections. Changes of this order after stimulation of the adrenal cortex have been reported in the literature (8, 9, 18). The mean value of these cells increased significantly in the adrenalectomized group of rats after adrenaline injections; but no change occurred after adrenaline treatment in the splenectomized or adrenalectomized-splenectomized groups. These data suggest that the spleen may have a role in the depression of the lymphocytes which is observed after adrenal cortical stimulation. The significant increase in the mean absolute number of lymphocytes after the adrenaline injections in the adrenalectomized group probably resulted from direct stimulation of the white pulp of the spleen in the absence of the adrenal cortical activity.

Polymorphonuclear Leucocytes per cu. mm. of Blood. A statistically significant increase occurred in the mean absolute number of circulating polymorphonuclear leucocytes following adrenalectomy, splenectomy, and following the adrenaline injections in the normal, the sham-operated normals, the adrenalectomized, and the adrenalectomized-splenectomized groups of rats. The fact that the dual operation of adrenalectomy-splenectomy did not result in a change of the mean value of these cells is in marked contrast to the increase which resulted after removal of the adrenals or spleen alone. This suggests that the adrenals and spleen are dependently related to a neutrophilic response in the rat. Support of this possible relationship is given by the evidence that there was no change in the mean value of these cells following adrenaline injections in the splenectomized group of rats.

Figure 2 shows the relationship of the polymorphonuclear leucocytes with the ascorbic acid concentration of fresh adrenal glands and the spleen of rats in the normal intact, adrenaline-treated, and saline-injected groups. Regression lines for the normal intact and adrenaline-injected rats shown in figure 2 are similar to the data reported for such animals in other experiments (19). Comparison of the adrenaline-injected group with the normal group shows that a definite polymorpho-leucocytosis in the former group is associated with a marked decreased level of ascorbic acid concentration of the adrenals but no change in the ascorbic acid concentration of the spleen. Data for the saline-injected rats indicates that they experienced some disturbance in this relationship as a result of the injections, but there is neither a leucocytosis nor significant change in the adrenal ascorbic acid. It is evident from data in figure 2 that the absolute number of polymorpho-leucocytes is not related to the splenic ascorbic acid concentration nor does this ascorbic acid participate or respond to adrenaline injections.

Regression of the polymorphonuclear leucocytes on the adrenal ascorbic acid

concentration in spleenectomized and sham-spleenectomized rats is shown in figure 3. The regression line for the sham-spleenectomized group is similar to that of the normal intact rats. The spleenectomized group has an obvious polymorpho-leucocytosis, but this is not associated with a change in the adrenal ascorbic acid concentration.

Tests for statistical differences in the mean ascorbic acid concentration of the adrenals and the spleens of these 5 groups of rats represented in figures 2 and 3 show that a significant difference (decrease) was induced in the adrenal ascorbic acid in the adrenaline-injected group alone. These data indicate that the polymorpho-

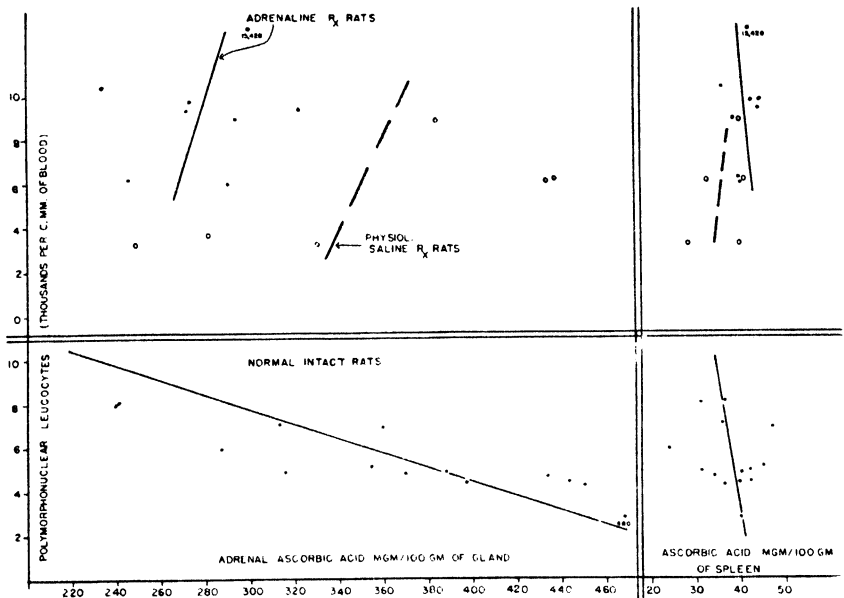


FIG. 2. REGRESSION OF POLYMPHONUCLEAR LEUCOCYTES per cu. mm. of blood on the ascorbic acid concentrations of adrenal glands and spleen of a group of normal intact rats, a group of rats 3 hours after the last of a series of adrenaline injections, and a group of control rats injected with physiological saline.

leucocytosis induced with adrenaline in normal rats is different in its mechanism from the leucocytosis which follows spleenectomy (table 1).

DISCUSSION

The data presented indicate that the polymorphonuclear leucocytosis which follows spleenectomy or adrenalectomy is related to processes which are essentially different from the polymorpho-leucocytosis which follows adrenaline injections. The latter is clearly associated with a change in the concentration of the adrenal ascorbic acid. The fact that the ascorbic acid concentration of the spleen was not altered by the injections of adrenaline suggests a specificity of reaction of this substance which is related to its site rather than the mere presence in an organ. It is well known that a

duced depression of the eosinophiles. This effect, which has been shown by Thorn's group to be an indication of adrenal cortical activity, was not altered when the rats were splenectomized. It is possible that the spleen may affect the course of the eosinophile response to adrenaline injections since a marked eosinophilia was induced in adrenalectomized rats but not in adrenalectomized-splenectomized rats.

It is believed that the role of the adrenal and spleen in the regulation of the leucocytic picture can be critically evaluated only when definite corticoid products are available for experimental use. However, the data presented have indicated

TABLE 1. ASCORBIC ACID CONTENT OF ADRENAL GLANDS AND SPLEEN OF NORMAL AND EXPERIMENTAL RATS

ANIMAL	ADRENAL ASCORBIC ACID MG/100 GM. FRESH GLAND	P ¹	SPLEEN ASCORBIC ACID MG/100 GM. FRESH SPLEEN	P
<i>Normal</i>				
a. Intact (14).....	362.6 ± 20.4 ²		37.0 ± 1.9	
b. Physiol. Saline (6).....	352.4 ± 32.5		36.9 ± 2.6	
(a, b).....		>0.5		>0.5
c. Adrenaline ³ -injected (8).....	279.6 ± 10.4		41.5 ± 1.0	
(a, c).....		>0.02		>0.05
<i>Operated</i>				
d. Sham splenectomized (8).....	379.2 ± 36.9			
(a, d).....		>0.5		
e. Splenectomized (7).....	408.5 ± 13.9			
(a, e).....		>0.1		

Number in parentheses indicates the number of rats.

¹ P is the value of the difference between the means occurring by chance alone. ² Mean ± S. E. of the Mean. ³ Adrenaline given s.c. at a dose level of 0.02 mg./100 gm. of body weight for 3 successive hourly intervals.

several points where the spleen and adrenal are closely related to the final blood picture which may evolve after certain experimental procedures.

SUMMARY

The effect of the adrenal cortex on certain aspects of the blood leucocyte picture has been shown to involve the participation of the spleen. Absence of the spleen prevents the typical response of the lymphocytes and neutrophils to adrenaline injections. This role of the spleen does not involve a change in its ascorbic acid concentration, nor the ascorbic acid concentration of the adrenal glands.

The depression of the eosinophiles following adrenal cortical stimulation with

adrenaline was not affected by the absence of the spleen. The eosinophile therefore seemed most unequivocally, of the leucocytes studied, responsive to adrenal cortical activity alone. A marked eosinophilia, however, can be induced with adrenaline in adrenalectomized rats but remain unaffected in number in adrenalectomized-spleenectomized rats.

Graphically represented in this paper is the relationship of the absolute number of polymorphonuclear leucocytes per cu. mm. of blood to the concentration of ascorbic acid in the adrenal glands and spleen of normal intact, adrenaline-injected, and saline-injected rats. The relation of the polymorphonuclear leucocytes to the adrenal ascorbic acid is also represented for a group of spleenectomized and sham-spleenectomized rats. These data show that the polymorpho-leucocytosis induced with adrenaline injections is related to a decreased ascorbic acid content of the adrenals, but this condition induced by spleenectomy is not associated with any change in the adrenal ascorbic acid concentration.

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EFFECT OF DESOXYCORTICOSTERONE ACETATE AND ADRENAL CORTICAL EXTRACTS ON SURVIVAL OF ADRENALECTOMIZED AND INTACT RATS AFTER BURNING¹

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WHEN resistance to various stresses is impaired by adrenalectomy, it has been generally considered improbable that the administration of desoxycorticosterone acetate (DCA) has any restorative effect (1, 2). On the other hand, after adrenalectomy the administration of adrenal cortical extract (ACE) appears to confer protection against certain forms of stress (1, 2). The experiments reported in this paper indicate that when DCA or ACE is given to rats for several days prior to burning, protection against burn shock is afforded by DCA but not by ACE. Evidence is presented also that administration of DCA to the intact rat can increase its resistance to burning.

With the exception of Hechter (3) who found that DCA could increase the bleeding volume of adrenalectomized rats, and Katz *et al.* (4) who reported a favorable effect on shock after venous thrombosis in intact dogs, most workers (2, 5, 6, 7) share the opinion that by itself DCA is ineffective in protecting against most shocking procedures, both in adrenalectomized and normal animals. Katz *et al.* (4) gave DCA for a period before producing the injury, while in most other experiments its administration has been therapeutic only. Results from the use of ACE in normal animals have varied; some authors reported protective effects (6-11), while others reported negative findings (5, 12).

That neither ACE nor DCA increased the resistance of non-adrenalectomized animals after burns has been reported by Rosenthal (13) in mice and by Ingle and Kuizenga (14) in rats, when these substances were given after burning. Our experiments were designed to show whether or not DCA or ACE given to adrenalectomized rats over a period prior to burning could increase resistance to a standard burn, and whether or not the same agents could increase the resistance of normal non-adrenalectomized rats to a more severe burning.

METHOD

Male and female rats (Wistar strain) weighing about 150 gm. were used in the experiments. They were kept in individual cages at a temperature of 24° to 27°C., and were fed Purina dog chow and water *ad libitum*. Food intake was noted but did not appear to be directly related to survival in these series.

After bilateral adrenalectomy the animals were given 0.9 per cent NaCl solution *ad libitum* for 5 to 14 days. At the end of this period they were divided into 3 groups,

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the first of which constituted a control and continued to receive saline. Desoxycorticosterone acetate (DCA)² or adrenal cortical extract (ACE)³ was administered subcutaneously to the second and third groups which were allowed water *ad libitum*. Injections were given once, or in the case of the higher doses of ACE, twice daily. After at least 5 to 8 injections a standard burn was produced under ether anesthesia by immersing the backs of all animals in water at 85°C. for 30 seconds. The last injections of the cortical preparations were given on the day of burning. The criterion of survival was that the animal should survive for at least 3 days after burning. The surviving animals could be maintained by administering saline. Withdrawal of saline resulted in death in all but 2 cases.

The degree of hemoconcentration was estimated in a group of adrenalectomized rats treated with DCA; hemoglobin values were determined by a photolorimetric method before and 2 hours after burning. These were compared with figures obtained from adrenalectomized controls. After the burn only one estimation was performed when blood was taken by cardiac puncture.

The effect of saline given by the intraperitoneal route was tested in another experiment. An injection of 5 cc. of 0.9 per cent saline was given to a group of adrenalectomized rats one hour before burning. This was repeated at the time of the burn, and again after 3 to 4 hours. A fourth injection was made after 24 hours if the animal was still living. In this experiment both the experimental and adrenalectomized control group were burned at 75°C. for 30 seconds.

DCA or ACE was administered to nonadrenalectomized rats prior to burning in a similar fashion. At the end of the injection period both treated and control groups were burned in such a manner that a majority of control rats would die within several hours. A series of trials carried out beforehand indicated that a temperature of 90°C. for 40 seconds fulfilled this requirement.

Three series of experiments using 93 rats of both sexes were carried out to study the effect of DCA, ACE and saline when these were given subsequent to burning. The procedure resembled that described above except that 3 or 4 mg. of DCA was given intramuscularly immediately after burning. ACE (Upjohn, 50 μ) or saline was given subcutaneously to appropriate groups in equivalent amounts (1 cc.) immediately after burning and again after 2½ and 5 hours. Control groups which received no treatment were included in 2 of the 3 series.

RESULTS

Results of the experiments on survival rate of adrenalectomized rats are summarized in table 1. The higher doses of DCA (0.5 mg., 1.0 mg.) afforded definite protection, while ACE showed little or no protective effect in the dosage employed. Although the potency of the 2 extracts was expressed in terms of dog units, and both are known to be effective clinically, it was felt that the concentration of factors of possible value in shock might differ. The figures obtained lend no support to this

² Percorten, Ciba.

³ Two extracts were used, one containing 50 μ /cc. prepared by the Upjohn Company, Kalamazoo, Michigan, and supplied through the kindness of Dr. D. J. Ingle, and the other containing 30 μ /cc. prepared by the Connaught Medical Research Laboratories, University of Toronto.

view as no difference in effect between the two preparations was demonstrated. There was no evidence that sex influenced the mortality rate nor the efficacy of treatment.

It was observed that the survival rate in the control group was higher when the interval between adrenalectomy and burning was long. However, in all instances, treated and control groups were burned after the same interval.

The intake of food and gain in body weight per day varied considerably, both within and between groups. The average daily intake was between 12 and 15 gm/rat in the different groups, yet the change in body weight was variable, ranging from an

TABLE 1. EFFECT OF DCA AND ACE ON SURVIVAL OF ADRENALECTOMIZED RATS AFTER BURNING¹

NO. OF RATS	TREATMENT	DOSAGE	NO. OF SURVIVALS
17	Saline	Oral	3
10	ACE	15-30 DU.	0
10	ACE	60 DU.	1
4	ACE	150 DU.	1
5	DCA	0.25 mg.	0
5	DCA	0.50 mg.	4
18	DCA	1.00 mg.	16

¹ The group receiving 150 DU of ACE were males and those receiving 0.25 mg. and 0.5 mg. of DCA were females. The sexes were distributed as evenly as possible in the other groups.

TABLE 2. EFFECT OF DCA ON HEMOCONCENTRATION AFTER BURNING IN ADRENALECTOMIZED MALE RATS (7 DAILY INJECTIONS OF 1 MG. PRIOR TO BURNING)

NO. OF RATS	TREATMENT	AVERAGE HB. BEFORE BURNING (GM. % \pm S.D.)	AVERAGE HB. 2 HR. AFTER BURN- ING (GM. % \pm S.D.)
10	DCA	12.6 \pm 1.5	16.6 \pm 1.8
10		13.8 \pm 0.7	17.4 \pm 0.9

TABLE 3. EFFECT OF SALINE INJECTIONS ON SURVIVAL TIME OF ADRENALECTOMIZED MALE RATS AFTER BURNING, 75°C., 30 SECONDS

NO. OF RATS	TREATMENT	DOSE IN 24 HR.	AVERAGE SURVIVAL TIME (HR.)
5			5.1 (3-8)
10	Saline	15-20 CC.	23.6 (4-56)

average loss of 1.2 gm. to a gain of 26.0 gm. (in one of the DCA groups) during the period of injections.

The beneficial effect of DCA, as judged by the mortality rates and the relatively greater gain in weight noted in one group treated with DCA (previously cited), suggested the possibility that water retention had occurred. In table 2 the effect of DCA on hemoglobin concentration is shown. The hemoglobin values before burning were lower in the DCA treated group than in the control group. The percentage rise in hemoglobin in the 2 hours following the burn was slightly larger in the treated group and it will be noted that the hemoglobin percentage approached that of the control group in the final estimate.

When saline was injected intraperitoneally in adrenalectomized rats both prior and subsequent to burning, the survival time was prolonged, but the percentage survival was not increased. These results are shown in table 3.

Results of experiments using non-adrenalectomized rats are shown in table 4. DCA when given daily for 5 to 7 days prior to burning exerted a favorable effect. ACE did not appear to be any more effective in the dosage used than an equivalent amount of saline.

When DCA, ACE, or saline was given to normal rats *subsequent* to burning, the results obtained were equivocal. In one of the 3 experiments ACE gave greater protection than saline or DCA, while in two, such an effect was not demonstrated. With DCA no favorable effect was shown. In their variability these findings do not differ from those reported in the literature.

DISCUSSION

A survey of the pertinent literature shows no uniformity of opinion as to the efficacy of ACE and of DCA in protecting adrenalectomized or normal animals from traumatic shock. Results of the experiments reported herein show that under our experimental conditions DCA has a protective effect against burn shock, both in the adrenalectomized and in the intact rat. Most of our experiments may be regarded as prophylactic. In cases where a significant effect occurred, the DCA had been admin-

TABLE 4. EFFECT OF DCA AND ADRENAL CORTICAL EXTRACT¹ ON SURVIVAL OF NON-ADRENALECTOMIZED RATS AFTER BURNING, 90°C., 40 SECONDS (7 DAILY INJECTIONS PRIOR TO BURNING)

NO. OF RATS	SEX	TREATMENT	DOSE	NO. OF SURVIVALS
10	M	Saline	1 cc.	2
10	M	ACE	1 cc.	3
10	M	DCA	1 mg.	6
10	F			0
10	F	DCA	1 mg.	10

¹ Upjohn, 1 cc. = 50 DU.

istered for some time before burning. The same favorable result did not occur when treatment was started after burning.

Some of the discrepancies in the literature may be explained in part by differences in experimental procedure. The species, the age, the previous condition of the animals used, the amount and type of diet administered, anesthesia, environmental temperature, and the degree of burning have all been shown by many workers to affect mortality rates to an important degree. These conditions as well as the experimental procedures have differed in nearly every experimental series. In our study the groups of rats compared have been similar in as many respects as possible and treated and untreated groups have been run concurrently throughout. A variation in one or more of the factors mentioned might quite possibly have resulted in inconclusive findings.

The relative ineffectiveness of ACE in the dosage used as compared to DCA deserves some comment, and again it is suggested that the variable factors inherent in this type of experiment played a large part. The prevailing view, for which excellent evidence exists, indicates that when given in suitable amounts ACE will protect adrenalectomized rats against many forms of stress (1). However, other investigators have usually chosen other types of trauma for study. When a burn of the type used

in the present study is produced, swelling takes place at the site of injury but no fluid is lost from the surface during the shock phase. It might be expected that the action of DCA on electrolyte balance might be more efficacious in such a case than would ACE. If the dose of ACE were increased, similar protection might be observed. Experiments in this laboratory have shown that when the dosage of ACE is increased considerably and injections are started a day previous to and are continued for 12 hours following burning (60 DU given every 2 hours), adrenalectomized animals could be protected against the same type of burn as was used in the experiments with DCA. This suggests that the dosage of ACE used in our earlier experiments was too low. The proper dosage of ACE in almost any condition is necessarily determined empirically.

The mechanism by which DCA acts in raising the resistance of both normal and adrenalectomized rats is not known. Katz *et al.* (4) suggested that in shock produced by massive venous thrombosis a decreased rate of fluid loss from the circulation accounted for its effect. This explanation is attractive, yet is probably not adequate to explain our findings, as the same (or greater) percentage increase in hemoglobin took place within 2 hours after burning in adrenalectomized rats whether DCA was administered or not. In addition to this, intraperitoneal saline injections did not protect adrenalectomized rats against the burn. Lowdon *et al.* (15) reported that the loss of serum sodium which occurred after scalds was restored by injecting DCA. Studies of electrolyte balance, especially with regard to changes in levels of sodium and potassium in body fluids may help to explain the protective action of DCA.

The role of the adrenal cortex in traumatic and burn shock still remains obscure. The possible relationships of electrolyte balance to the circulatory changes and to the metabolic effects which occur after injury are complex and require much further work.

SUMMARY

Desoxycorticosterone acetate (DCA) administered in daily doses of 1 mg. to adrenalectomized rats for 5 to 8 days prior to burning protected them against a burn usually lethal in control animals. Among the 18 animals thus treated 16 survived the burn while in 17 control adrenalectomized rats only 3 survived. No significant difference in the degree of hemoconcentration 2 hours after burning was noted between adrenalectomized controls and adrenalectomized animals treated with DCA. Adrenal cortical extracts (ACE) injected into 24 adrenalectomized rats at daily dose levels up to 150 DU for 5 to 8 days before burning resulted in only 2 survivals.

DCA was found to raise the resistance of non-adrenalectomized rats against a more severe burning, whereas ACE did not show a similar effect. Eighteen of 20 untreated rats died after the burning while of 20 rats, which received daily injections of DCA (1 mg.) for 7 days prior to burning, 16 survived. When ACE or DCA was given after burning variable and inconclusive results occurred. Neither substance appeared to be any more efficacious than were small amounts of saline.

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MECHANISM OF EXTRACELLULAR SODIUM AND CHLORIDE DEPLETION IN THE ADRENALECTOMIZED DOG¹

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THE mechanism of extracellular sodium and chloride depletion in adrenal insufficiency has long been disputed. One concept supported by the work of Loeb *et al.* (1) and Harrop and his co-workers (2) is that sodium and chloride loss may be attributed solely to renal wastage with resultant extracellular dehydration. The opposing view, held chiefly by Swingle and his collaborators (3-5) is that the hormone of the adrenal cortex controls the normal distribution of sodium, chloride and water between intra- and extracellular fluid compartments and that during adrenal insufficiency the total loss of sodium and chloride from the extracellular compartment is far greater than can be accounted for by excretory loss of these ions. A third theory (6-8) states that hypotonicity of the extracellular fluids in adrenal cortical insufficiency is the result of increased renal excretion of sodium and chloride and that therefore a secondary shift of water into cells occurs.

The evidence in support of these views has been largely inferential. Strict evaluation of the mechanism of extracellular Na and Cl depletion requires a knowledge of *a*) the total amounts of Na and Cl in the extracellular compartment and *b*) the Na and Cl balance of the animal during adrenal insufficiency. Previously Harrop (8) and Clarke *et al.* (9) approached an answer to this problem having measured plasma ionic concentrations, blood volume and 'extracellular' fluid with SCN. They did not, however, carry out concomitantly the balance studies necessary to reach definitive conclusions.

Thus we undertook a determination of the total 'extracellular' sodium and chloride in *a*) normal dogs, *b*) bilaterally adrenalectomized dogs maintained on desoxycorticosterone acetate and *c*) dogs in acute adrenal insufficiency. These determinations together with sodium and chloride balance studies made it possible to test whether the 'extracellular' sodium and chloride depletion in acute adrenal insufficiency could be accounted for by excretory loss alone.

MATERIALS AND METHODS

Seven normal male dogs were adrenalectomized in two stages under open drop-ether anesthesia using a lumbo-dorsal retroperitoneal approach. Two weeks to two

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² Lt. (j.g.), M.C. U.S.N. Views or opinions expressed in this paper are not necessarily those of the Navy Department.

months were allowed between the first and second stages. These animals were maintained on 1.5 mg/day of desoxycorticosterone acetate³ intramuscularly and an approximately one per cent sodium chloride diet for about 18 days. At this time the animals were apparently normal as judged by general appearance, activity, blood urea nitrogen, blood sugar and plasma electrolyte concentrations. Desoxycorticosterone acetate was then withdrawn and the animals were allowed to develop severe adrenal insufficiency characterized by muscular weakness, prostration, refusal of food, elevated blood urea nitrogen and elevated plasma potassium.

Four of the animals were treated with intravenous NaCl and glucose and recovered from the crisis. Two of these animals were subjected to two complete cycles of insufficiency (1F and 19F). Two animals died with hypoglycemic convulsions during maintenance on DCA following recovery from crisis and their tissues were analyzed for Na, Cl, K and water (5F and 3B). Four animals (1F, 7F, 14F, 19F) were allowed to die in adrenal insufficiency in order that tissue ionic analysis could be made.

Sodium, Potassium and Chloride Balance Studies. Balance studies were made throughout three periods: *Period I*, begun on the day of the second adrenalectomy and terminated on the last day of DCA administration; *Period II*, begun on the first day of DCA withdrawal and ended on the day of acute adrenal insufficiency; *Period III*, begun on the day of resumption of DCA therapy and terminated when DCA was again discontinued. Animals were placed in steel wire metabolism cages for the duration of balance periods and were removed only for weighing or for measuring blood and 'extracellular' fluid volumes.

Food. Animals were fed Purina Dog Chow which was first pulverized in 2-kg. lots, mixed well, and aliquots analyzed for sodium, potassium and chloride. Dogs in adrenal insufficiency have rather capricious appetites so that any food not eaten within two hours was collected and analyzed along with feces.

Urine. Urine was collected under mineral oil in 24-hour periods. At the end of each balance period the sides and bottoms of the cages were scrubbed with a steel wire brush and were rinsed with 1500 cc. distilled water. The washings were analyzed for sodium, potassium and chloride and the totals added to that of the urine collected on the last day of the balance period.

Feces. Feces were collected in tared air-tight tins for 7 days at a time; the total was mixed well with a large power-driven beater and an aliquot was analyzed for sodium, potassium, chloride and water. Feces for each balance period were crudely separated into periods by feeding 0.5 gm. carmine on the first day of each new balance period. Animals dying in acute adrenal insufficiency were immediately autopsied and the total contents of the gastrointestinal tract analyzed for sodium, potassium and chloride.

Determination of Fluid Compartments. Simultaneous determinations of thiocyanate space (10), mannitol space, and plasma volume by T-1824 (11) were made at the beginning and end of each balance period. Mannitol space was determined by injecting a known amount of a 15 per cent solution of mannitol⁴ from a calibrated syr-

³ Cortate, Schering: Desoxycorticosterone acetate in sesame oil kindly supplied by the Schering Corporation, Bloomfield, N. J.

⁴ Kindly supplied by Sharp & Dohme, Inc.

inge into the external jugular vein. Forty minutes were allowed for equilibration with the 'extracellular' fluids (12-14). Then 5 blood samples were drawn at 10-minute intervals from the opposite jugular vein and the plasma mannitol concentration was determined by the method of Corcoran and Page (15). The serial plasma concentrations were then plotted against time on semilogarithmic graph paper and the volume of distribution of mannitol was calculated from these data in the usual manner.

Ionic Determinations. Sodium and potassium concentrations of plasma, erythrocytes, urine, feces, food and tissues were determined by internal standard flame photometry after the methods of Overman and Davis (16) using the Perkin-Elmer Model 52A Flame Photometer. Chloride was analyzed chemically by a modification of the Volhard method (17). Total water for each of these substances was determined by drying to constant weight at 110° C. (usually required 72 hours for tissues).

Preparation of Tissues for Analysis. Tissues were prepared for analysis by the method of Overman and Davis (18). In the case of normal dogs death from respiratory failure was produced in 30 seconds to 2 minutes by intravenous injection 1.3 gm. pentobarbital. Brain, skeletal muscle, liver, heart and spleen were immediately excised, trimmed free of gross fat and connective tissue and sponged free of external blood. Each sample was immediately weighed and placed in a Waring blender with a known amount of 1 N nitric acid containing lithium as an internal standard for flame photometry. The sample was homogenized and allowed to extract in lusteroid tubes overnight in the refrigerator. The supernatant was cleared by centrifugation and then filtration and an aliquot of the supernatant was analyzed for Na⁺, K⁺, and Cl⁻. Tissues of dogs in adrenal insufficiency or in hypoglycemia on maintenance DCA were prepared similarly except that these animals, when moribund, were placed on a dog board and the withdrawal of 25 ml. of blood sufficed to precipitate death.

Blood Sugar and Urea N. Blood urea nitrogen concentration was determined by direct Nesslerization (19) and blood sugar by the method of Folin and Wu (20) using a photoelectric spectrophotometer.

Calculations. The total 'extracellular' sodium and chloride were calculated as the sum of: a) Total plasma Na⁺ and Cl⁻ = plasma volume × plasma (Na) and (Cl). b) Total erythrocyte Na⁺ and Cl⁻ = circulating red cell mass (from plasma volume and centrifuge hematocrit) × red cell (Na) and (Cl). c) Total 'interstitial' Na⁺ and Cl⁻ = 'Interstitial' fluid volume × calculated 'interstitial' (Na) and (Cl). 'Interstitial' fluid volume = SCN space - blood volume. 'Interstitial' (Na⁺) and (Cl⁻) were calculated after the formula used by Manery and Hastings (21):

$$(\text{Na})_E = 0.95 \times (\text{Na})_S \quad (\text{Cl})_E = \frac{(\text{Cl})_S}{0.95}$$

where (Na)_E is the sodium concentration of the interstitial fluid, (Na)_S is the sodium concentration of the serum water (calculated from plasma (Na), plasma water, and plasma specific gravity, 22), and 0.95 is the Gibbs-Donnan equilibrium ratio between mammalian serum and its ultrafiltrate.

The total erythrocyte sodium and chloride were considered as part of the 'extracellular' ions because it was found in our experiments and in those of others (23)

that the changes in concentration of sodium and chloride in the dog erythrocytes reflect the concentration changes occurring in the plasma.

TABLE 1. FLUID VOLUMES AND IONIC CONCENTRATION IN DOGS WITH ACUTE ADRENAL INSUFFICIENCY

DOG NO.	CONDI- TIONS OF EXPER.	WT.	HCT. VOL.	PLASMA			ERYTHROCYTE			PLASMA SPEC. GRAVITY	PLAS- MA H ₂ O	PLAS- MA VOL.	BLOOD VOL.	EFV/ KG. SCN	EFV/ KG. (MAN- NITOL)
				Na	K	Cl	Na	K	Cl						
		kg.	%	mEq/l.	mEq/l.	mEq/l.	mEq/l.	mEq/l.	mEq/l.		%	kg.	kg.		
1F	A	10.2	45	135	3.7	110	110	5.7	69.5	1.028	92.0	49	89	304	286
	B	7.3	33	140	4.4	114	118	4.9	69.2	1.0235	92.5	58	87	395	372
	C	6.6	36	125	6.4	101	106	6.2	69.2	1.028	91.5	47	75	322	334
	D	6.8	25	144	3.9	118	118	5.1	59.0	1.0265	93.0	64	86	350	
	E	5.9	31	124	6.8	93	105	8.1	55.0	1.030	91.5	39	57	280	
7F	A	11.8	53	147	4.2	108	119	6.3	57.0	1.025	92.2	56	120	292	
	B	10.7	42	145	4.2	120	115	5.2	63.0	1.0245	92.3	66	114	332	352
	C	9.6	54	132	7.4	109	111	5.6	60.2	1.029	91.0	35	77	346	332
5F	A	9.6	46	139	3.7	112	110	5.7	60.0	1.023	92.0	58	85	282	236
	B	10.7	44	152	4.4	112	135	5.7	71.2	1.024	92.0	62	115	277	298
	C	8.8	39	132	5.3	91	107	6.0	60.2	1.027	91.0	39	56	256	201
	D	8.6	28	146	4.6	118	116	6.5	69.0	1.026	92.7	79	108	490	396
3B	A	11.1	46	149	3.9	109	126	5.7	79.0	1.027	92.0	49	91	282	273
	B	11.1	40	149	3.8	112	129	6.3	72.0	1.025	93.0	45	75	379	350
	C	10.4	52	125	7.6	98	104	7.8	59.0	1.0285	91.5	38	78	268	292
	D	10.4	42	139	5.0	102	109	7.2	56.0	1.026	92.5	44	76	380	316
1B	A	9.6	43	149		117	130	6.8	68.0	1.028	92.5	63	109	250	252
	B	11.3	29	146	3.9	112	120	6.3	69.0	1.026	93.2	80	110	405	289
	C	9.0	33	130	5.6	106	107	7.2	64.0	1.033	90.8	73	108	318	344
14F	A	10.9	39	149	4.0	107	122	5.3	57	1.027	91.5	55	89	274	254
	B	10.4	32	152	4.7	106	119	5.5	59	1.026	92.0	74	107	259	266
	C	9.0	43	135	6.0	100	107	5.9	54	1.031	91.5	41	71	228	
19F	A	7.7	40	144	4.2	104	115	5.0	60.3	1.0285	93.5	63	104	315	
	B	8.2	42	144	3.8	108	115	4.7	62.0	1.0275	93.0	51	78	346	
	C	7.4	44	128	6.5	95	115	6.5	61.0	1.032	91.0	40	72	290	
	D	6.3	39	138	4.0	102	118	5.5	60.0	1.030	91.5	46	75	302	
	E	5.9	37	138	5.5	110	114	6.8	64.0	1.0305	90.0	35	57	171	

A = Control. B = Bilaterally adrenalectomized maintained on DCA 1.5 mg/day. C = First acute adrenal crisis. D = Recovery from crisis maintained on DCA 1.5 mg/day. E = Second acute adrenal crisis.

RESULTS

Determinations of fluid compartment volumes and plasma and erythrocyte ionic concentrations are presented in table 1. In 5 dogs, simultaneous measurements were made of the so-called 'extracellular' fluid volume using thiocyanate and mannitol because in certain febrile states SCN has been shown to enter cells and its volume of

distribution to approach the value for total body water (24). In the controls the average SCN 'space' was 3000 cc. or 28 per cent of the body weight while the average volume of distribution of mannitol was 2700 cc. or 26 per cent of body weight. Bilaterally adrenalectomized dogs maintained on DCA showed an average SCN space of 3400 cc. or 35 per cent of the body weight and the values obtained with mannitol were identical. Animals in acute adrenal insufficiency showed a SCN 'space' of 2500 cc. or 30 per cent of the body weight whereas the mannitol 'space' was 2600 cc. or 31 per cent of the body weight. Thus under these experimental conditions, just as in certain other patho-physiological states (25), mannitol and thiocyanate are apparently diluted by approximately the same fluid volume.

Blood and Plasma Volume. Bilaterally adrenalectomized dogs maintained on DCA show an average increase in plasma volume of 9 per cent; whereas the blood volume remains fairly constant. This is obviously accompanied by a decrease in the volume of packed red cells and indicates an actual reduction in the total amount of circulating red cells. During acute adrenal insufficiency there is a marked reduction (average of 28%) in plasma volume as has been previously reported (9). This is accompanied by a smaller decrease in blood volume and some degree of hemoconcentration. Marked hypotension and the fact that withdrawal of as little as 20 cc. of blood may often produce death within a few seconds in the more severely insufficient animals reflect the profound oligemia seen in the acute adrenal crisis.

'Extracellular' Fluid Volume. Bilaterally adrenalectomized dogs maintained on 1.5 mg/day of DCA and approximately one per cent salt diet show an average increase of 13 per cent in absolute SCN volume and a 25 per cent increase when expressed on the basis of body weight. That this is a *real* increase in 'extracellular' fluid and not merely a reflection of weight loss can be determined when we consider that normal animals losing 29 per cent of the body weight from starvation show the following changes in fluid compartments:

a) Absolute volumes (26):

T-1824: 29 per cent *decrease*; Na SCN: 15 per cent *decrease*.

b) Fluid volumes per kg. body weight (26):

T-1824: 2 per cent *increase*; Na SCN: 17 per cent *increase*.

If the apparent expansion of the SCN space in the treated dogs were due to weight loss we would expect a decrease in the absolute SCN volume rather than the *increase* which actually occurs.

As might be expected, animals in acute adrenal crisis show an average reduction of 26 per cent in SCN space, but at the same time they lose about 13 per cent of their body weight, so that, expressed as percentage of body weight, the SCN space decreases only 14 per cent on the average. These data agree with those of Harrop (8) and Clarke *et al.* (9). It will be noted that there is little or no change in SCN space per kg. in adrenal insufficiency when compared to that of control animals.

Ionic Balance. Table 2 illustrates ionic balance data on one animal (19F) which is representative of the group. These data are in qualitative accord with those of Loeb (2) and Harrop (1) but differ quantitatively in that our animals generally showed smaller excretory losses of sodium and chloride. The consensus in the literature is that there is a rather marked retention of potassium by the kidneys during adrenal

insufficiency (27), but our animals usually showed only small positive potassium balances or even appreciably negative potassium balances.

TABLE 2. FLUID AND IONIC BALANCE STUDIES IN ACUTE ADRENAL INSUFFICIENCY (*Dog 19F*)

PERIOD	DAY	WT.	Na ⁺ INTAKE	Na ⁺ OUTPUT	Na ⁺ BALANCE	K ⁺ INTAKE	K ⁺ OUTPUT	K ⁺ BALANCE	Cl ⁻ INTAKE	Cl ⁻ OUTPUT	Cl ⁻ BALANCE
		kg.	mM	mM	mM	mM	mM	mM	mM	mM	mM
I	1	7.7	42.9	31.6	+11.3	55.8	39.7	+16.1	34.2	29.4	+4.8
	2	7.8	14.3	19.2	-4.9	18.6	25.8	-7.2	11.4	23.7	-12.3
	3	7.9	28.6	8.2	+20.4	37.2	22.3	+14.9	22.6	10.0	+12.6
	4	7.8	28.6	10.4	+18.2	37.2	26.4	+10.8	22.6	6.7	+15.9
	5	8.0	28.6	8.4	+20.2	37.2	27.9	+9.3	22.6	22.5	+0.1
	6	8.1	28.6	12.9	+15.7	37.2	34.8	+2.4	22.6	22.6	0.0
	7	8.2	28.6	19.7	+8.9	37.2	29.0	+8.2	22.6	22.8	-0.2
	8	8.4	28.6	11.8	+16.8	37.2	19.1	+18.1	22.6	10.6	+12.0
	9	8.6	30.8	11.7	+19.1	37.2	31.3	+5.9	29.8	16.2	+13.6
	10	8.6	30.8	42.7	-11.9	37.2	32.9	+4.3	29.8	34.5	-4.7
	11	8.5	15.4	13.5	+1.9	18.6	22.7	-4.1	14.9	12.2	+2.7
	12	8.4	30.8	17.2	+13.6	37.2	22.5	+14.7	29.8	18.1	+11.7
	13	8.3	15.4	11.8	+3.6	18.6	21.5	-2.9	14.9	49.1	-34.2
	14	8.2	15.4	19.1	-3.7	18.6	26.7	-8.1	14.9	21.3	-6.4
	15	8.2	30.8	73.9	-43.1	37.2	28.7	+8.5	29.8	52.8	-23.0
II	1	7.9	15.4	52.3	-36.9	18.6	15.4	+3.2	14.9	48.5	-33.6
	2	7.6	25.5	74.7	-49.2	27.2	23.9	+3.3	21.9	63.0	-41.1
	3	7.5	20.2	47.8	-27.6	23.6	24.7	-1.1	14.0	42.3	-28.3
III	1	7.4	52.8	13.2	+39.6	11.8	39.0	-27.2	49.7	26.4	+23.3
	2	7.3	10.1	19.7	-9.6	11.8	24.6	-12.8	7.0	14.6	-7.6
	3	7.2	10.1	22.2	-12.1	11.8	20.9	-9.1	7.0	13.6	-6.6
	4	6.9	23.8	14.1	+9.7	18.9	21.4	-3.5	9.3	7.2	+2.1
	5	6.8	13.7	18.7	-5.0	7.1	7.3	-0.2	2.3	15.1	-12.8
	6	6.9	27.2	10.5	+16.7	11.8	11.9	-0.1	24.1	5.4	+18.7
	7	6.7	27.2	3.0	+24.2	11.8	6.5	+5.3	24.1	1.2	+22.9
	8	6.5	30.8	4.4	+26.4	7.1	9.5	-2.4	19.8	2.2	+17.6
	9	6.4	30.8	17.0	+13.8	7.1	11.1	-3.4	19.8	15.2	+4.6
	10	6.4	30.8	12.7	+18.1	7.1	10.5	-3.4	19.8	18.8	+1.0
	11	6.3	27.2	3.2	+24.0	11.8	4.8	+7.0	24.1	2.0	+22.1
IV	1	6.2	22.1	6.7	+15.4	5.9	6.4	-0.5	20.6	4.0	+16.6
	2	6.1	22.1	35.7	-13.6	5.9	12.1	-6.2	20.6	25.9	-5.3
	3	6.0	22.1	39.0	-16.9	5.9	14.5	-8.6	20.6	29.3	-8.7
	4	5.9	0	0	0	0	0	0	0	0	0

I = Bilaterally adrenalectomized DCA 1.5 mg/day. II = DCA discontinued and crisis developed. III = Recovery on DCA 1.5 mg/day. IV = DCA discontinued and second crisis developed.

It is conceivable that with tissue destruction taking place during adrenal insufficiency, potassium liberated from dying and dead cells elevates the plasma potassium; but at the same time excretion of potassium, although impaired, is still sufficient to produce a negative potassium balance. Too, these differences may be due to the

fact that our animals were maintained on DCA after adrenalectomy; while those of Loeb and Harrop were either untreated (2) or treated with whole adrenal cortical extract (1).

For purposes of comparing total 'extracellular' sodium and chloride losses with excretory loss of these ions we have separated the experimental animals into two groups. Table 3 presents average data for 7 dogs during their first adrenal crisis. Three of these died during the crisis (7F, 1B, 14F). These animals showed a marked drop in 'extracellular' Na^+ (35%) and Cl^- (32%) during the development of acute adrenal insufficiency. But during this period the net loss of Na^+ and Cl^- was not sufficient to account for the total 'extracellular' loss. Thus, on the average, 117 mm of sodium and 66 mm of chloride apparently disappeared from the 'extracellular'

TABLE 3. NATIVE IONIC BALANCES IN SEVEN DOGS

	Na	Cl
<i>Total 'Extracellular' Ions</i>		
1. Adrenalectomized dogs on DCA (1.5 mg/day for 18 days).....	479 mm	385 mm
2. Acute adrenal insufficiency.....	310 mm	248 mm
Total 'extracellular' loss.....	169 mm	137 mm
<i>External Ionic Balance</i>		
1. Adrenalectomized dogs on DCA		
Intake.....	261 mm	214 mm
Output.....	143 mm	138 mm
Balance.....	+118 mm	+77 mm
2. Acute adrenal insufficiency		
Intake.....	149 mm	123 mm
Output.....	201 mm	192 mm
Balance.....	-52 mm	-69 mm
Total 'extracellular' loss in acute insufficiency.....	169 mm	137 mm
External balance (intake-output).....	-52 mm	-69 mm
'Extracellular' ionic loss unaccounted for	117 mm	66 mm

fluid and could not be accounted for by excretory loss of these ions. Dog 7F was an exception to the trend found in the other animals. In his case, the total reduction in 'extracellular' Na and Cl could be completely accounted for by excretory loss of these ions. Two animals (table 4) demonstrated during their second bout of adrenal insufficiency changes similar quantitatively to the other group.

Four animals which were treated during insufficiency and recovered from the first crisis (table 5) showed during recovery a total 'extracellular' gain of 154 mm sodium and 120 mm chloride. At the same time the net gain of sodium from food was only 144 mm and of chloride 95 mm. Even more striking are the findings in the case of dog 3B (table 6). In this animal 164 mm more sodium and 95 mm more chloride were lost from the 'extracellular' fluid than were lost by excretion. During recovery,

TABLE 4. NATIVE IONIC BALANCES IN TWO DOGS DURING SECOND ADRENAL CRISIS (1F, 19F)

	Na	Cl
<i>Total 'Extracellular' Ions</i>		
1. Adrenalectomized dogs recovering from crisis (DCA 1.5 mg/day for 10 days).....	295 mM	243 mM
2. Second adrenal crisis.....	175 mM	139 mM
Total 'extracellular' loss.....	120 mM	104 mM
<i>External Ionic Balance</i>		
1. Adrenalectomized dogs recovering from crisis		
Intake.....	275 mM	220 mM
Output.....	100 mM	108 mM
Balance.....	+175 mM	+112 mM
2. Second adrenal crisis		
Intake.....	128 mM	114 mM
Output.....	138 mM	129 mM
Balance.....	-10 mM	-15 mM
Total 'extracellular' loss in acute insufficiency.....	120 mM	104 mM
External balance (intake-output).....	-10 mM	-15 mM
'Extracellular' ionic loss unaccounted for.....	100 mM	89 mM

TABLE 5. NATIVE IONIC BALANCE IN FOUR DOGS (3B, 5F, 1F, 19F) DURING RECOVERY FROM ADRENAL INSUFFICIENCY

	Na	Cl
<i>Total 'Extracellular' Ions</i>		
1. Adrenalectomized dogs in adrenal insufficiency.....	297 mM	233 mM
2. Recovery on DCA.....	451 mM	359 mM
Total 'extracellular' gain.....	154 mM	126 mM
<i>External Ionic Balance</i>		
1. Adrenalectomized dogs recovering from acute adrenal insufficiency		
Intake.....	208 mM	176 mM
Output.....	64 mM	81 mM
Balance.....	+144 mM	+95 mM
Total 'extracellular' gain during recovery.....	154 mM	126 mM
External balance (intake-output).....	144 mM	95 mM
'Extracellular' ionic gain unaccounted for.....	+10 mM	+31 mM

this animal gained back 166 mM sodium and 93 mM chloride from other than exogenous sources. In this case the fluid volumes were measured at the sixtieth hour of

recovery from crisis and thus before the animal had much time to gain exogenous sodium and chloride. From these data it is inferred that sodium and chloride are being added to the 'extracellular' fluid during recovery from adrenal insufficiency and that the source of these ions is endogenous.

Tissue Analysis. Inferential evidence that during adrenal insufficiency most of the sodium and chloride which is lost from the 'extracellular' fluid is not actually

TABLE 6. NATIVE IONIC BALANCE DURING DCA ADMINISTRATION, ACUTE ADRENAL INSUFFICIENCY AND RECOVERY

	Na	Cl
<i>Total 'Extracellular' Ions</i>		
1. Adrenalectomized on DCA (1.5 mg/day for 18 days).....	627 mM	468 mM
2. Acute adrenal insufficiency.....	368 mM	275 mM
Total 'extracellular' loss.....	259 mM	193 mM
3. Acute adrenal insufficiency.....	368 mM	275 mM
4. 60th hour of recovery on DCA 1.5 mg/day.....	583 mM	420 mM
Total 'extracellular' gain.....	215 mM	145 mM
<i>External Ionic Balance</i>		
1. Acute adrenal insufficiency		
Intake.....	377 mM	331 mM
Output.....	472 mM	429 mM
Balance.....	-95 mM	-98 mM
Total 'extracellular' loss insufficiency.....	259 mM	193 mM
External balance (intake-output).....	-95 mM	-98 mM
'Extracellular' ionic loss unaccounted for.....	164 mM	95 mM
2. Recovery on DCA		
Intake.....	68 mM	66 mM
Output.....	19 mM	14 mM
Balance.....	+49 mM	+52 mM
Total 'extracellular' gain on recovery.....	215 mM	145 mM
External balance.....	49 mM	52 mM
'Extracellular' ionic gain unaccounted for.....	166 mM	93 mM

absent from the body led us to analyze some soft tissues of animals dying in adrenal insufficiency and of animals bilaterally adrenalectomized and maintained on DCA.

Control data are presented for 7 normal animals (table 7). In adrenal insufficiency the muscle intracellular water is presumably increased (7) and the concentration of intracellular solids thus reduced. There is, therefore, little rationale for expressing tissue ionic concentrations in terms either of intracellular water or intracellular solids if a comparison with normal is desired. Consequently tissue ionic concentrations here are expressed on the basis of wet tissue weight since this is the material actually

analyzed. Sufficient data are presented to allow calculations of ionic concentrations in other terms.

It can readily be seen (table 7) that normal tissue ionic concentrations vary so greatly from dog to dog that all but extreme variations in ionic levels in experimental animals fall within the normal range. Analysis of tissues from dogs in adrenal insufficiency revealed no deviation from the normal range with the exception of tis-

TABLE 7. TISSUE IONIC CONCENTRATIONS AND WATER CONTENT OF SEVEN NORMAL DOGS

	WATER CC. PER KG. WET TISSUE	Na mm PER KG. WET TISSUE	Cl- mm PER KG. WET TISSUE	K mm PER KG. WET TISSUE
Brain				
Average.....	775	60.0	40.9	94.9
Low.....	759	52.5	37.7	87.4
High.....	787	68.5	46.7	108.5
Muscle				
Average.....	757	26.3	15.7	100.8
Low.....	740	23.9	13.7	97.0
High.....	785	29.1	17.6	106.0
Liver				
Average.....	739	52.1	37.9	72.7
Low.....	720	43.9	33.4	63.5
High.....	782	60.9	46.4	83.0
Heart				
Average.....	775	38.9	26.5	78.7
Low.....	765	32.6	21.6	67.7
High.....	787	46.5	29.2	87.7
Spleen				
Average.....	757	61.4	48.7	62.2
Low.....	740	59.0	47.5	50.0
High.....	780	83.3	51.7	94.9
	WATER CC/KG. PLASMA	SPECIFIC GRAVITY PLASMA	PLASMA Na, meq/L.	PLASMA Cl- meq/L.
Plasma				
Average.....	922	1.0244	150	110
Low.....	902	1.0210	140	105
High.....	937	1.0265	156	115

sue water. Only in heart and skeletal muscle was there a consistent increase in water content.

Tissue sodium and chloride concentration was found to be slightly decreased on the average but was still within the normal range. In the case of muscle, where chloride is presumed to be extracellular and thus the chloride space (28) is an approximation of extracellular fluid volume, the decrease in sodium and chloride concentration can be attributed to the decreased concentrations of these ions in the extracellular fluid. The calculated 'excess Na' of muscle (29) is actually decreased in adrenal insufficiency when compared to normals.

The concentration of potassium in the tissues is either very slightly decreased or normal when expressed on the basis of wet tissue weight. However, when expressed on the basis of dry solids, potassium concentration is found to be increased. This calculation agrees with that of Darrow *et al.* (7).

TABLE 8. TISSUE IONIC CONCENTRATIONS AND WATER CONTENT OF FOUR DOGS IN ACUTE ADRENAL INSUFFICIENCY

TISSUE	DOG NO.	WATER GM/KG. WET TISSUE	Na MM/KG. WET TISSUE	Cl- MM/KG. WET TISSUE	K MM/KG. WET TISSUE
Brain	1F	765	53.4	36.7	87.2
	7F	768	55.5	39.5	90.3
	14F	772	53.5	38.4	90.5
	19F	765	59.7	39.2	85.2
Muscle	1F	788	18.3	14.1	84.4
	7F	787	23.1	16.0	103.0
	14F	790	23.9	16.3	102.0
	19F	785	27.5	20.8	87.5
Liver.....	1F	763	45.4	36.4	76.4
	7F	763	55.3	41.9	64.7
	14F	765	42.5	35.8	91.0
	19F	750	53.5	45.0	70.5
Heart	1F	786	39.0	31.1	70.0
	7F	792	27.8	29.0	75.2
	14F	795	33.2	26.3	93.0
	19F	779	40.5	34.0	75.5
Spleen	1F	777	43.7	43.6	88.2
	7F	748	31.9	29.5	62.6
	14F	782	47.0	43.5	90.0
	19F	770	41.0	44.0	94.5
		WATER GM/KG. PLASMA	SPECIFIC GRAVITY PLASMA	PLASMA Na meq./L.	PLASMA Cl- meq./L.
Plasma	1F	910	1.0295	126	106
	7F	907	1.0295	132	107
	14F	915	1.0280	135	97
	19F	900	1.0305	138	110

Of the conditions studied tissue ionic concentrations are most greatly altered in adrenalectomized animals maintained on DCA (table 9). With the exception of heart muscle, there is little change in water content of the tissues studied. The rather striking increments in sodium and chloride concentrations in skeletal muscle and heart have been previously reported (30-32). Buell and Turner (30) showed these changes occurred to a greater degree in adrenalectomized rats than in normal rats treated with DCA. There is also a striking reduction in muscle and heart potassium.

DISCUSSION

The analyses presented offer further evidence that the total 'extracellular' depletion of sodium and chloride during adrenal insufficiency is not due solely to excretory loss of these ions. An interesting corollary finding is that 4 animals in adrenal insufficiency, subsequently treated with DCA and intravenous saline and glucose, regained part of the lost extracellular sodium and chloride from some source other than food or administered salt. This is clearly shown by the data for *dog 3B* (table 6). Early in the course of recovery from adrenal insufficiency (60th hour) it would seem that the animal gained back from endogenous sources an amount of sodium and chloride almost equivalent to the amount which disappeared from the extracellular

TABLE 9. TISSUE IONIC CONCENTRATIONS AND WATER CONTENT OF TWO DOGS BILATERALLY ADRENALECTOMIZED AND MAINTAINED ON DCA

TISSUE	DOG NO.	WATER CC/KG. WET TISSUE	Na mm/KG. WET TISSUE	Cl- mm/KG. WET TISSUE	K mm/KG. WET TISSUE
Brain	5F	772	67.4	62.0	94.9
	3B	770	74.0	58.0	87.5
Muscle	5F	760	64.6	44.5	65.0
	3B	770	59.2	36.4	73.0
Liver	5F	762	57.4	48.9	91.0
	3B	760	64.6	65.3	67.6
Heart	5F				
	3B	800	65.0	55.4	71.0
Spleen	5F	741	49.8	54.7	108.8
	3B	752	64.9	57.4	83.8
		WATER CC/KG. PLASMA	SPECIFIC GRAVITY PLASMA	PLASMA Na meq/L.	PLASMA Cl- meq/L.
Plasma	5F	930	1.021	162	118
	3B	935	1.020	152	132

fluid during previous acute insufficiency. It might be postulated that the reason the other animals did not show a *quantitatively* similar gain during recovery is that measurements were made later in the course of recovery, 4th to 10th day. By this time the action of DCA had caused sodium and chloride entrance into cells of soft tissues and had thus produced a markedly positive sodium and chloride balance which masked the 'extracellular' Na^+ and Cl^- gain from endogenous sources.

The analyses of brain, muscle, liver, heart and spleen of dogs in adrenal insufficiency do not indicate sodium and chloride entrance into the cells of these tissues and, at least in the case of skeletal muscle, the calculated 'intracellular' sodium concentration is actually decreased. Further, the work of Rigler (33) on adrenalectomized rats showed sodium and chloride concentrations of skin and subcutaneous tissues were reduced.

On the other hand in adrenalectomized animals treated with DCA there is a marked increase in the concentration of Na and Cl of muscle, and the calculated 'intracellular' Na^+ is increased indicating some Na entrance into cells. A few simple calculations show that a 10 kg. bilaterally adrenalectomized dog treated with DCA has 4 kg. of skeletal muscle (40% of body weight). This 4 kg. of muscle contains 248 mm Na^+ , of which 186 mm is 'extracellular' (calculated from chloride space). When this dog is in acute adrenal insufficiency he will weigh about 9 kg. and have 3.6 kg. of skeletal muscle. This muscle will contain only 86 mm Na^+ , of which 68 mm is 'extracellular' and 18 mm 'intracellular.' Thus in addition to the large decrease in 'extracellular' Na^+ there is about a 44 mm decrease in 'intracellular' sodium. This adds further to the amount of sodium which apparently 'disappears' during adrenal insufficiency and is unaccounted for by excretory loss.

If the data presented be accepted as a qualitative indication of the direction of ion movement in experimental adrenal insufficiency in the dog, the problem resolves itself into a search for endogenously 'lost' extracellular Na and Cl. Since it has been estimated from the work of Harrison, Darrow and Yannet (28) that the 'excess' Na of bone represents approximately 20 to 25 per cent of the total body Na, it is not unreasonable to postulate that bone Na may, in part, represent a labile body store for this ion. It may be theorized that in adrenal insufficiency, Na leaves the extracellular fluid compartment by *a*) the renal route and *b*) by being sequestered in bone and that during recovery, the sequestered Na may be mobilized and re-appear in the extracellular fluid drawing with it water from the soft tissues.

Technical difficulties encountered in the accurate measurement of bone Na have to date prevented a critical test of this hypothesis. Radioactive sodium (Na^{24}) may prove a valuable tool in the solution of this problem.

SUMMARY

Approximations were made of the total 'extracellular' sodium and chloride of control dogs, adrenalectomized dogs on DCA and dogs in acute adrenal insufficiency. A comparison of the 'extracellular' sodium and chloride loss during adrenal insufficiency with the total excretory loss of these ions showed more sodium and chloride lost from the extracellular compartment than could be accounted for by excretion alone. Furthermore, during recovery from adrenal insufficiency animals gained back more 'extracellular' sodium and chloride than could be accounted for by gain of these ions from food. Analysis of soft tissues in adrenal insufficiency agrees with previous work of others and suggests that sodium and chloride do not enter the cells of these tissues in adrenal insufficiency. An hypothesis is proposed suggesting that bone may serve as a labile 'store' for sodium in adrenal insufficiency.

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PRODUCTION OF DIABETES IN THE MOUSE BY PARTIAL PANCREATECTOMY¹

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THE mouse has become the animal of choice for certain experiments involving radioactive substances available only in very limited quantities. Recently Lazarow (1) and Waisbren (2) showed that mice can be made diabetic by intravenous injection of alloxan. In order to avoid the possibility of kidney and liver damage caused by alloxan, it was desirable to produce a diabetic mouse by partial pancreatectomy.

In the mouse as in the rat, the diffuse character of the pancreas makes a complete pancreatectomy impossible. Overholser (3) found that the head portion of the pancreas, which is attached to the duodenum, contains the least amount of islet tissue, while the mid-portion of the pancreas has the highest islet tissue content. Because of this distribution, a pronounced diabetes can be produced in rats by a careful partial pancreatectomy in which the duodenal portion is not entirely removed because of possible damage to the duodenal circulation and the bile duct. Shapiro and Pincus (4) removed the greater portion of the rat pancreas by electrocautery. Ingle and Griffith (5) reported the removal of much of the rat pancreas by picking away the tissue with forceps. It was found in this laboratory (Pauls and Drury (6)) that definite diabetes could be produced in the rat by a more rapid procedure which entailed a smaller mortality rate. This was done by aspiration of the pancreatic tissue by means of a glass tip connected to a vacuum line. This method was adapted to the production of diabetes in mice.

METHODS

Adult albino mice which had not been fasted were used. Under continuous ether anesthesia and sterile conditions, a midline incision was made extending from the xiphoid process for about two centimeters posteriorly. The animal was then draped with a gauze sponge which had been slit appropriately. Retraction was done either by an eye speculum or by S-shaped wires which had been flattened on one end and attached by the opposite end to rubber bands thumbtacked to the operating board. As in the case of rats (6), aspiration only was used for the removal of the pancreatic tissue. For mice, a glass tube was drawn to a tip of 0.7 mm. inside diameter and was attached to a trap and filter pump assembly. It was usually found necessary to use the maximum vacuum developed by the water pump. By experience it was possible

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to adjust the negative pressure and the size of the suction tip so that the pancreatic tissue could be removed without rupturing major blood vessels embedded there-in.

The stomach was grasped very gently with ring forceps and the portion of the pancreas along the greater curvature removed by suction with a minimum of bleeding. With the stomach held back out of the field, the next portion removed was that lying between the duodenum, transverse colon and the spleen. Great care was exercised to remove the pancreatic tissue without rupturing the splenic veins. With the duodenum greatly reflected to the left of the animal, a portion of the head of the pancreas lying between the duodenum and the lesser curvature of the stomach was removed. Because of the necessity for leaving the bile duct intact, only that portion lying between it and the duodenum was removed, care again being taken that the veins and arteries supplying the gut be left. In the mouse it was not always feasible to remove the portion lying dorsally near the portal vein and in the region of the lymph nodes. The hemorrhage of small vessels at any period during the operation was allayed by pressure with sponges moistened in warm saline. Following the removal of most of the pancreas, except that lying between the bile duct and the lesser curvature of the stomach, the incision was closed in two layers with continuous silk sutures. Coating the incision with flexible collodion prevented gnawing of the sutures and the danger of infection.

TABLE 1. HIGH CARBOHYDRATE DIET ON WHICH PARTIALLY DEPANCREATIZED MICE WERE MAINTAINED

Sucrose.....	60%	Salt mix ¹	4%
Casein.....	18%	Water.....	8%
Yeast.....	10%		

¹Osborn-Mendel.

Post-operative care included keeping the animals warm and protected from drafts because of their well-known susceptibility to respiratory infections. The mice were maintained on a stock diet for a week while they recovered from the operation. They were then placed on a high carbohydrate diet (table 1) for a week or longer, at the end of which time they were put in small metabolism cages for 24 hours. Urine sugars and volumes were determined as well as blood sugar levels. These determinations were used as an index for the degree of diabetes that had developed. The amount of sugar excreted in urine was determined by the Shaffer-Somogyi titration method (7) and the blood sugars were determined by the Folin-Malmros micro method (8) using 0.1 ml. of blood collected from the tail.

RESULTS

Because of the small size of the mice and the delicacy of their tissues, it was found that a certain amount of acquired skill in performing the pancreatectomies was necessary for a reasonable number of mice to survive the operations. The amount of negative pressure applied and the size of the suction tip were found to be critical factors. A seemingly small amount of hemorrhage resulted in a fatal loss of blood if the splenic or duodenal vessels were ruptured. Necessary post-operative care included keeping the animals warm and out of drafts.

The blood sugar and urine sugar and volume data for the group of mice that

TABLE 2. BLOOD SUGAR LEVELS IN PARTIALLY DEPANCREATIZED MICE MAINTAINED ON HIGH SUGAR DIET

MOUSE NUMBER	DAYS POST-OPERATION	BLOOD SUGAR	MOUSE NUMBER	DAYS POST-OPERATION	BLOOD SUGAR
		mg. %			mg. %
11	24	350	61	15	206
	25	335		18	196
12	24	410	68	15	162
	25	410		18	165
15	24	400	69	15	450
				18	575
37	45	205		20	675
46	31	340			
	34	500			

TABLE 3. GLUCOSE EXCRETION IN 24 HOURS BY PARTIALLY DEPANCREATIZED MICE ON HIGH SUGAR DIET

MOUSE NUMBER	DAYS POST-OPERATION	BODY WEIGHT	URINE VOLUME	GLUCOSE EXCRETED
		gm.	ml/24 hr.	mg/24 hr.
11	37	16	15	1090
12	22	14	33	2360
	37	14	43	3490
15	22	15	30	2200
20	14	18	6	340
37	21	26	9	745
	22	26	9	566
	23	26	7	370
46	20	25	15	1090
	21	25	27	1900
	22	25	20	860
61	9	24	8	688
	11	23	6	388
68	8	17	6	684
	9	17	7	712
	11		7	518
	12		9	930
69	9	13	12	1270
	11	13	33	1720
	12		18	1860
70	8	11	10	766
	9		6	1096
	11		17	975

survived the operation and developed a diabetic condition are shown in tables 2 and 3. When the surviving mice were fed the high carbohydrate diet, an adequate removal of the pancreas was shown by a marked polyuria and glycosuria and a high blood sugar level. Diabetic mice were found to maintain severe glycosuria and polyuria for as long as two months until killed for other experiments. During this period no insulin was administered. In these respects they resemble rats more than other experimental diabetic animals. Unoperated control mice and operated mice which retained more than about 10 per cent of the pancreas showed neither glycosuria nor polyuria while maintained on the high carbohydrate diet.

Mice which excreted 24-hour urine volumes of over 6 ml. or approximately twice the normal value showed a significant amount of excreted glucose, as well as blood sugar levels of at least 160 mg. per cent. The degree of polyuria was proportional to the amount of glycosuria.

The intensity of the diabetes developed in mice is indicated by values for *mouse 12* which excreted three times its body weight in urine over a period of 24 hours. With this it put out 3.49 grams of glucose or the equivalent of 249 grams per kilogram. Other mice put out 2 to 2.5 liters of urine per kilogram body weight during a 24-hour period. Blood sugar values as high as 675 mg. per cent were recorded. These figures are considerably higher than the comparable values measured for diabetic rats produced by the same means (6). Reports of blood sugars in mice made diabetic by alloxan injections (1, 2) are not so high as the highest of our series. No reports of urine volumes and sugars in the alloxan mice were given.

It may be concluded that a definite diabetes can be produced in the mouse by surgical removal of approximately 90 per cent of the pancreas. It is more severe than that produced by alloxan in the mouse. On a high carbohydrate diet, the severity of the diabetes increased with post-operative survival time.

SUMMARY

A pronounced diabetes was produced in the mouse by the aspiration of approximately 90 per cent of the pancreas. The glycosuria and polyuria so produced was very marked. The animals survived for several weeks without remission of symptoms and without insulin injections.

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IMPAIRMENT OF LIVER GLUCONEOGENESIS FROM LACTIC ACID AS A RESULT OF ALLOXAN ACTION

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HOUSAY and coworkers (1) proved that alloxan, when injected into dogs just after pancreatectomy, causes hypoglycemia of the same order as when injected into normal animals, thus showing that the mechanism of alloxan hypoglycemia is largely, if not totally, independent of any insulin that might be released from pancreatic islands under alloxan action. The same conclusion was reached, through a different experimental approach (2, 3). The fact that when diabetes has been established, either by pancreatectomy or by alloxan, this drug fails to cause a blood sugar lowering, and possibly other facts remain to be explained (4, 5), but these do not invalidate the evidence afforded by the occurrence of alloxan hypoglycemia under different experimental conditions in all of which there can be no question of insulin being released by the pancreas under alloxan action. It has been suggested that alloxan hypoglycemia probably results from some direct action of the drug upon the liver, and results previously reported (2, 3) very strongly support this suggestion.

Cori and Cori (6) found that the hyperglycemia elicited by the subcutaneous injection of epinephrine, 200 $\mu\text{g}/\text{kg.}$, to 24-hour fasted rabbits lasts for not less than 4 hours, and evidence presented in this paper shows that intramuscular injections of similar or even smaller doses of this substance also cause blood sugar to rise and remain above normal for not less than 4 hours (See Tables 2C and 3A). It is likewise well known that intravenous injection of the usual diabetogenic doses of alloxan into 24-hour fasted rabbits causes hyperglycemia of a few hours duration. It might therefore have been expected that, when about 200 mg. of alloxan per kg. were given intravenously to 24-hour fasted rabbits one hour after the intramuscular injection of epinephrine, 200 $\mu\text{g}/\text{kg.}$, the resulting hyperglycemia would be prolonged and increased by alloxan action; or would, at least, be as long lasting as when similar rabbits are subject to no further treatment after the epinephrine injection. Contrary to this expectation, when studying the influence of a previous injection of epinephrine upon the effect of alloxan in rabbits, it was found that, in many cases 2 hours after alloxan, and only 3 hours after epinephrine, there was extreme hypoglycemia. A possible explanation for the failure of alloxan to cause any further rise in blood sugar under the circumstances could be that, at the time of alloxan injection, the liver had been deprived of most of its glycogen by the previous action of epinephrine. But this does not account for the existence of hypoglycemia at a time when epinephrine alone would be acting to keep blood sugar above normal. The possibility suggested itself, therefore, that some effect of alloxan had blocked the mechanism by means of which hyperglycemia is sustained for some hours after an hypodermic or intramuscular injection of epinephrine in the fasting rabbit. This mechanism is generally agreed to be the so-called Cori cycle (7). The hypothesis was therefore considered that some effect of the initial action of alloxan might prevent muscle glycogen from being eventually changed into blood sugar through this cycle, by interfering either with 1) muscle glycolysis, 2) liver glycogenolysis, or 3) liver gluconeogenesis from lactic acid. The second is most unlikely, since the first clear-cut effect of alloxan action upon carbohydrate metabolism is hyperglycemia, which, as previously shown (8), is accompanied by a considerable fall in liver glycogen and a noteworthy increase in liver dextrose, which must mean that glycogenolysis is not hindered,

but enhanced, by the early action of alloxan. Glycolysis in muscle extracts from frogs is inhibited by alloxan *in vitro* (9). Inhibition of muscle glycolysis by alloxan action is quite compatible with impairment of liver gluconeogenesis from lactic acid. This last possibility appeared to us as particularly worthy of inquiry.

The experiments described herein were accordingly planned to study liver capacity for gluconeogenesis from fed lactic acid in fasted rabbits after alloxan injections and in other experimental conditions.

METHODS

In order to appraise liver capacity to build glycogen from lactic acid, it seemed desirable to work with 24-hour fasted animals, in which low and not too widely divergent liver glycogen levels might be expected, and also to make lactate available to the liver in rather considerable amounts, so as to ascertain whether or not any significant increase in liver glycogen from fasting levels was unquestionably caused by gluconeogenesis from lactic acid.

Besides a few experiments that were not completed, brief mention of which is made in the discussion, a total of 30 rabbits was employed. After some days under uniform feeding, the animals were fasted for 24 hours and subsequently subjected to one or other of different experimental procedures. The details of these are stated in the table headings.

Lactic acid was given by stomach tube. The racemic 85 per cent C.P. acid was either totally or only half-neutralized with sodium hydroxide and diluted with distilled water up to a 5 or 10 per cent solution. Epinephrine was given by intramuscular injection, from Parke Davis ampuls of 1:1,000 adrenalin hydrochloride. Alloxan was administered by intravenous injection, as a 5 per cent solution in distilled water of alloxan monohydrate.¹ Blood sugar was estimated in blood samples from the marginal ear vein by the method of Folin and Wu, as modified by Andes and Northup (10). At the end of each experiment, or for rabbits 1, 2, 3, and 4 at the end of the 24-hour fasting period without further treatment (table 1), when the animal was fully anesthetized with sodium pentobarbital, the abdomen was opened and 2 suitable samples of liver quickly withdrawn for glycogen estimation by the method of Good, Kramer and Somogyi (11). The liver glycogen figures given are the averages of satisfactory double determinations.

RESULTS AND DISCUSSION

Results of the experiments are presented in tables 1 to 3. Four experiments satisfied us that after a 24-hour fast, for the type of rabbits at our disposal and under our laboratory conditions, liver glycogen values were of the same order as those generally found in this species under similar circumstances (table 1, Group A). Such values are known to be rather widely variable, but despite this drawback, the difference between the fasting liver glycogen figures and those found after lactate feeding when no alloxan was injected is significant enough to warrant definite conclusions.

Since Cori and Cori (7) had shown that racemic lactic acid is a much less efficient liver glycogen builder than the D-form, it was thought desirable to feed a com-

¹ Alloxan monohydrate was kindly synthesized for us by Dr. Dora T. Garcia-Banus, from the Laboratorio de Investigaciones de Química Orgánica de la universidad los Andes.

paratively high dose and, accordingly, in the earlier experiments racemic lactic acid, 3 gm. per kg. of body weight, was administered. This dose, a 10 per cent solution of the unneutralized acid, killed a few animals, with severe injuries to the gastric mucosa

TABLE 1. LIVER GLYCOGEN IN 24-HR. FASTED RABBITS WITHOUT TREATMENT, OR 3-HR. AFTER LACTATE FEEDING (30 CC/KG., 10% SOLUTION OF TOTALLY NEUTRALIZED LACTIC ACID), WITH OR WITHOUT A PREVIOUS ALLOXAN INJECTION (200 MG/KG.)

A. FASTING			B. LACTATE FEEDING WITHOUT ALLOXAN			C. LACTATE FEEDING AFTER ALLOXAN		
No.	Weight	Liver glycogen	No.	Weight	Liver glycogen	No.	Weight	Liver glycogen
	gm.	%		gm.	%		gm.	%
1	1650	0.35	5	1350	1.45	8	1000	0.16
2	1900	0.80	6	1720	2.10	9	850	0.20
3	1800	0.60	7	870	1.10	10	1630	0.68
4	1350	0.75						

TABLE 2. BLOOD SUGAR AND LIVER GLYCOGEN VALUES 4 HR. AFTER LACTIC ACID-LACTATE FEEDING (30 CC/KG. OF 5% LACTIC ACID HALF NEUTRALIZED WITH NaOH) IN 24-HOUR FASTED RABBITS

NO.	WEIGHT	BLOOD SUGAR VALUES				LIVER GLYCOGEN
		Before	1 hr. after	2 hr. after	4 hr. after	
<i>A. Lactic acid-lactate feeding only</i>						
	<i>gm.</i>	<i>mg./100 cc.</i>				<i>%</i>
11	1800	106		107	114	4.80
12	1600	96		94	92	1.38
13	1550	110		107	112	2.10
<i>B. Alloxan directly before lactic acid feeding</i>						
14 ¹	1700	106	150	91	55	0.66
15 ¹	1000	104	212	248	244	0.97
16 ¹	1200	105	200	246	198	0.42
17 ²	1164	105	192	246	300	0.38
18 ²	864	110	149	174	106	0.41
<i>C. Epinephrine directly before lactic acid feeding</i>						
19 ³	1000	110	195	173	105	2.80
20 ³	1750	105	160	180	120	2.20
21 ³	1000	87	208	194	113	1.85
22 ⁴	1670	120	234	290	159	1.10
23 ⁴	895	108	188	250	143	1.82
24 ⁴	890	110	296	148	143	2.36

¹ Alloxan: 200 mg/kg. ² Alloxan: 300 mg/kg. ³ Epinephrine: 50 µg/kg. ⁴ Epinephrine: 100 µg/kg.

as found at post-mortem examination. Therefore, the acid was neutralized with sodium hydroxide, the same dose and dilution of the totally neutralized acid being used. Three lactate feeding experiments of this type (table 1, Group B) gave liver

glycogen figures markedly higher than those found in the fasting controls (table 1, Group A), showing that liver gluconeogenesis from lactic acid had taken place. When similar lactate feeding was immediately preceded by alloxan injection (table 1, Group C), liver glycogen figures were found of the same order as, or lower than, those found in the fasting controls. This might be interpreted as proving that alloxan had in some way interfered with liver capacity to build glycogen from lactic acid, thus substantiating the working hypothesis; but there was the possibility that the results might be accounted for by the glycogenolysis elicited by the initial alloxan action. It was therefore necessary to compare the effect of alloxan upon liver glycogen after lactic acid feeding with that of some other agent, such as epinephrine, equally capable of causing liver glycogenolysis. In addition, 2 rabbits that had been fed 3 gm/kg. of completely neutralized lactic acid had died after exhibiting violent con-

TABLE 3. BLOOD SUGAR AND LIVER GLYCOGEN VALUES 3 HR. AFTER LACTIC ACID-LACTATE FEEDING (30 CC/KG OF A 5% SOLUTION) OF LACTIC ACID HALF NEUTRALIZED WITH NaOH IN 24-HR. FASTED RABBITS

NO.	WEIGHT	BLOOD SUGAR VALUES				LIVER GLYCOGEN
		Before	2 hr. after	4 hr. after	7 hr. (3 hr. after lactic acid)	
<i>A. Epinephrine injection (300 µg/kg.) 4 hr. before lactic acid feeding</i>						
	gm.	mg./100 cc.				%
25	1172	75	270	154	80	3.23
26	1537	90	280	200	105	2.90
27	2000	115	310	304	120	2.60
<i>B. Alloxan injection (200 mg/kg.) 4 hr. before lactic acid feeding</i>						
28	1380	90	155	60	40	0.70
29	1390	98	160	72	55	0.94
30	1500	102	154	80	60	0.84

vulsions and without hypoglycemia. This result suggested the possibility that such toxic action might be due to alkalosis, brought about by such a high dose of sodium lactate. The effect of a smaller dose of only half-neutralized lactic acid was then tried. A further reason for using a smaller dose of lactate was that it was desirable to follow the effect of either epinephrine or alloxan upon blood sugar, and for this reason it became necessary to give lactic acid in doses lower than 2 gm/kg., since, as previously shown (12), higher doses produce by themselves an increase in blood sugar in fasting rabbits. It also seemed desirable to let one more hour elapse after the lactate feeding before the liver glycogen estimation.

Three experiments (table 2A) warrant the conclusion that 1.5 gm/kg., of half neutralized lactic acid given by stomach tube does not cause any important change in blood sugar, or any other noteworthy disturbance in fasted rabbits, and that such lactic acid-lactate feeding is followed, at the end of 4 hours by liver glycogen values markedly higher than those found in fasting rabbit controls (table 1, Group A). By

comparing the figures in table 2*B* and *C*, it is very clear that, despite the glycogenolysis that must have preceded the blood sugar rise caused by epinephrine, the liver glycogen values 4 hours after epinephrine injection and lactic acid feeding are much higher than those found 4 hours after alloxan injection and similar lactic acid feeding. Again, this might be interpreted as showing that alloxan, besides causing liver glycogenolysis, which is known to result from epinephrine as well as alloxan injections, in opposition to what happens with epinephrine, prevents or considerably hinders liver gluconeogenesis from lactic acid, thus bearing out the working hypothesis. The doubt occurred, nevertheless, that such results might not be conclusive inasmuch as liver glycogen level 4 hours after either epinephrine or alloxan might be mainly dependent upon the magnitude of glycogenolysis; and this, under these experimental conditions, might be greater in the alloxan than in the epinephrine experiments.

On the assumption that the magnitude of liver glycogenolysis is induced by either alloxan or epinephrine bears a quantitative relation to the magnitude of the resulting hyperglycemia, a comparison may be drawn between the average blood sugar and liver glycogen values following the injection of either alloxan or epinephrine for each of the experimental conditions. A comparison of the figures for *rabbits* 19, 20 and 21 and *rabbits* 22, 23 and 24 shows that in the epinephrine experiments, a higher dose and a higher hyperglycemia correspond to a lower liver glycogen, which may probably be related to a greater glycogenolysis. To the tentative conclusion, from the data of table 2*B* and *C* that alloxan action hinders gluconeogenesis from lactic acid, it might therefore be objected that liver glycogen levels in the alloxan experiments were lower than those in the epinephrine series, not because of a lesser gluconeogenesis but because of a greater glycogenolysis. That this objection is not sound is suggested by the fact that, in the alloxan experiment, the higher dose, with lowest liver glycogen, corresponds to a blood sugar average of the same order as, and a liver glycogen average markedly lower than, the corresponding figures for those rabbits, which received the smaller dose of epinephrine. For those with the lower alloxan dose, the hyperglycemia average is certainly higher than the corresponding figures for both epinephrine groups, and this might be supposed to account for the lower liver glycogen. But it should be noted that *rabbit* 14, under experimental conditions identical to those of *rabbits* 15 and 16, whatever may have been the degree of glycogenolysis elicited by alloxan, the 2-hour blood sugar value was no longer above normal, and the 4-hour sample was markedly low. Despite the lack of any indication of a specially great glycogenolysis in this case, in which there were no convulsions, the liver glycogen value (table 2*B*) was much lower than any of those found in the epinephrine experiments (table 2*C*). In the authors' opinion such low liver glycogen should be accounted for, not by particularly intense glycogenolysis but by impairment of gluconeogenesis. On the other hand, those rabbits receiving the higher alloxan dose exhibit the lowest liver glycogen average; this is well in accordance with the hypothesis of inadequate gluconeogenesis, whereas it cannot be accounted for by a greater glycogenolysis, since the hyperglycemia average in this group is of about the same order as in the epinephrine experiments.

From the foregoing discussion it appears that the contention that alloxan initial action in some way hinders liver gluconeogenesis from lactic acid remains the most

plausible conclusion to be drawn from the figures in table 2*B* and *C*. Nevertheless, it seemed worth while to search for still less questionable evidence. For this purpose it was desirable to test liver ability to build glycogen from lactic acid in animals having already undergone the glycogenolytic action of either alloxan or epinephrine, by feeding lactic acid 4 hours after the injection of either substance, i.e. after the glycogenolytic action, as judged from blood sugar levels, had entirely subsided in the alloxan experiments and was at least declining in the epinephrine series. In order to rule out the slight possibility that doses of epinephrine of 50 or 100 $\mu\text{g}/\text{kg}$. might be insufficient to cause a glycogenolysis of at least the order of magnitude of that induced by alloxan in the dosage used here, the dosage of epinephrine in these new experiments was raised to 300 $\mu\text{g}/\text{kg}$.; this resulted in hyperglycemias (table 3*A*) by no means smaller than those observed in our previous alloxan experiments (table 2*B*) and distinctly higher than those observed in the new alloxan experiments (table 3*B*). Blood sugar values 3 hours after lactate feeding showed that all glycogenolytic action was probably extinct in the alloxan treated animals, (table 3*B*) and extremely slight, if existent at all, in those receiving epinephrine (table 3*A*).

The liver glycogen figures show that when lactic acid was fed to 24-hour fasted rabbits 4 hours after the intramuscular injection of epinephrine, 300 $\mu\text{g}/\text{kg}$., 3 hours later the liver had built a considerable amount of glycogen from lactic acid. (Compare tables 1, Group A and 3*A*.) On the contrary, when lactic acid was fed to similarly fasted rabbits 4 hr. after the intravenous injection of 200 mg. of alloxan per kg., 3 hours later the liver glycogen was found to be much lower than following epinephrine (table 3*A*) and only slightly and perhaps not significantly higher than in fasting controls (table 1, Group A). In these experiments the lower level of liver glycogen in the alloxan-injected animals cannot be caused by a greater glycogenolysis, since, at the time of lactic acid feeding, so far as it can be judged from the magnitude of hyperglycemia, not only had glycogenolysis come to an end in the alloxan-injected animals, but also it had been much more marked in the epinephrine-injected animals. Unquestionably, these last experiments showed that liver gluconeogenesis from lactic acid was much smaller in the alloxan-injected than in the epinephrine-injected rabbits, and this result proves that some effect of the early action of alloxan does interfere with liver gluconeogenesis from lactic acid.

Under the conditions of our experiments, liver gluconeogenesis from lactic acid does not seem to be totally abolished. In the first place, 3 or 4 hours after lactic acid feeding, liver glycogen levels in our alloxan-injected rabbits (tables 1, Group C, 2*B* and 3*B*) were of about the same order as those found in fasting controls (table 1, Group A). Since alloxan injection causes by itself a lowering of liver glycogen (8), this means that some new formation must have taken place after, and probably from, lactic acid feeding. Also the fact that, with similar doses of alloxan, blood sugar values within 4 hours after the injection were, as a rule, distinctly higher when lactic acid was fed directly after the alloxan injection (table 2*B*) than when such feeding took place 4 hours later than the injection (table 3*B*) means, in the authors' opinion, that in the former case some of the fed lactic acid was changed first into liver glycogen and then into blood sugar. On the other hand, that the importance of this process was rather slight in the alloxan-injected animals is indicated by the facts that: 1) lactic

acid administration directly after alloxan did not prevent the early occurrence of hypoglycemia in *rabbit 14*, and 2) lactic acid feeding 4 hours after alloxan injection, when blood sugar was distinctly below the initial level (*rabbits 28, 29, and 30*), did not prevent a further degree of hypoglycemia, even though such development was apparently slower than might have been expected without lactic acid feeding.

In any event, the results reported in tables 1 and 2 *A, B, C* suggest and those in table 3 *A and B* prove that the early action of alloxan on 24-hour fasted rabbits very markedly hinders liver gluconeogenesis from lactic acid.

There can be little doubt that liver failure to build glycogen from lactic acid at a normal rate is the main factor in the genesis of alloxan hypoglycemia when a previous injection of epinephrine has caused both liver glycogenolysis and muscle glycolysis, as in previous experiments (2). Under other and more usual circumstances, other factors must be equally, or even more important. It is not known whether alloxan causes muscle glycolysis, as epinephrine does; other workers (13) found no increase in blood lactic acid after alloxan injection, and this would seem to indicate that it probably does not increase to any noteworthy degree. As already stated, it is known that it may even inhibit muscle glycolysis *in vitro* (9), and the possibility must therefore be considered that the same may be true *in vivo*. Gluconeogenesis from different non-carbohydrate sources, if only it would occur at the same rate as in normal fasting and resting animals, should be effective in preventing hypoglycemia, unless there be an increased dextrose utilization, which is not known to occur under alloxan action. It is therefore most likely that gluconeogenesis, not only from lactic acid but from any source, is more or less impaired by the early action of alloxan, and this may be the main factor in the production of alloxan hypoglycemia in most experimental conditions.

It should be pointed out that the hypothesis that a disturbance of liver gluconeogenesis is the main factor in the mechanism of alloxan hypoglycemia is not incompatible with the contention that it is caused by insulin released as a result of destructive action upon the islands of Langerhans. In fact, it seems to be well established that impairment of liver gluconeogenesis is an important factor in the mechanism of insulin action (14). But it must not be forgotten that, as stated at the beginning of this paper, alloxan hypoglycemia has been proved to occur under circumstances when no insulin can be released by the pancreas. Impairment of gluconeogenesis as a factor in the mechanism of alloxan hypoglycemia must therefore be a direct result of alloxan action upon the liver.

SUMMARY

In 24-hour fasted rabbits, 3 or 4 hours after lactic acid feeding, when this was directly preceded by alloxan injection, liver glycogen was much lower than when alloxan was not injected. In 24-hour fasted rabbits, 4 hours after lactic acid feeding, directly preceded by epinephrine or alloxan injections, liver glycogen was much lower in the alloxan- than in the epinephrine-injected animals. In 24-hour fasted rabbits, 3 hours after a lactic acid feeding which was preceded by either epinephrine or alloxan injections 4 hours earlier, liver glycogen was much lower in the alloxan- than in the epinephrine-injected animals. In these alloxan experiments just referred

to, lactic acid feeding did not prevent, but appeared to slow down the progress of alloxan hypoglycemia. In 24-hour fasted rabbits, when lactic acid was fed either directly after or 4 hours after an alloxan injection, liver glycogen was not, as a rule, significantly lower than in fasted but otherwise untreated controls. In 24-hour fasted rabbits injected with alloxan, when lactic acid was fed directly after the injection, the initial hyperglycemia was of greater magnitude than when lactic acid feeding was delayed for 4 hours.

It is concluded that the usual diabetogenic doses of alloxan, through some effect of its action upon the liver, for some time hinder, but may not totally abolish liver gluconeogenesis from lactic acid.

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EFFECTS OF LIGATION OF THE PANCREATIC DUCT UPON THE ACTION OF SECRETIN AND PANCREO- ZYMIN IN RABBITS WITH A CORRELATED HISTOLOGICAL STUDY

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THE histological study of the pancreas after ligation of the pancreatic duct has been described by various authors (1-4). However, the corresponding functional state, in so far as the external secretion is concerned, after ligation of the pancreatic duct has not been investigated. Previous work (5) has shown that secretin acts as a stimulus for increasing the volume rate of pancreatic juice secretion and pancreozymin for enzyme output of the pancreas. This study was undertaken in order to determine the effects of ligation of the pancreatic duct upon the action of secretin and pancreozymin in rabbits. A corresponding histological and histochemical examination of the pancreatic tissue was also undertaken.

METHODS

Acute experiments were carried out on rabbits, the main pancreatic ducts of which were ligated from 12 hours to 2 weeks previously. All these rabbits weighed 2.5 to 3.0 kg. and were anesthetized with an intravenous injection of sodium pentobarbital (32 mg/kg. body weight). Prolonged anesthesia was maintained when necessary by additional intravenous injections. The animals were fasted 24 to 48 hours before the experiments. A cannula was placed in the left femoral vein for secretin infusion. To preclude stimulation of the pancreas by acid or by bile in the duodenum, the pylorus and the common bile duct were occluded. Pancreatic juice was collected by a cannula inserted into the dilated main pancreatic duct proximal to the ligature.

By means of a perfusion pump, highly purified secretin dissolved in saline was continuously injected at the moderate rate of 0.03 mg/kg/hr. into the left femoral vein of the animal. The secretin preparation¹ was about 20 times as potent as SI. (6). The pancreozymin used in the present work was prepared by the aniline precipitation procedure (6) and showed no vasodepressor effect. The pancreatic juice collected during the first hour of response to secretin was discarded (5). The pancreatic juice was collected in 60-minute portions, in graduated centrifuge tubes on ice, and was stored in the refrigerator overnight. Two samples were usually collected before and 2 after the injection of pancreozymin. Pancreozymin was injected in a single dose of 10 mg. during the response to a constant dose of secretin.

After these samples were taken, the constant secretin infusion was stopped and a

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¹ This secretin was kindly supplied by Dr. E. D. Campbell, Eli Lilly and Company, Indianapolis.

period of 30 minutes was permitted to elapse. Then, the effect of a single supra-maximal dose of 0.5 mg. secretin was observed. The volume of pancreatic juice collected within 30 minutes after the injection was used as another measurement of the functional state of the pancreas.

The samples collected before and after the administration of pancreozymin during the constant secretin infusion were assayed for amylase content by the modified Willstatter method described in detail by Schmidt, Greengard and Ivy (7). The amylolytic activity unit was expressed in milligrams of maltose per cubic centimeter of juice as derived from the thiosulfate titration. From the amylase content of the samples, the output of this enzyme per hour was calculated by multiplying the concentration in mg/cc. by the volume of the sample in cc.

At the end of the experiment, the pancreatic tissue was removed and fixed in a modified fixing reagent described by Kristal (8) for histological study. In some experiments, the pancreatic tissues were fixed in cold acetone and studied histochemically for lipase and alkaline phosphatase according to Gomori's methods (9,10).

RESULTS

The effects of ligation of the pancreatic duct upon the volume and the amylase output of pancreatic secretion in response to constant secretin administration in 25 experiments is shown in figure 1. Four normal rabbits without ligation of the pancreatic ducts were used as controls. The hourly secretion of pancreatic juice falls from an average of 1.15 cc. in the controls to 0.61 cc. 12 hours after ligation and 0.47 cc. 24 hours after the operation. Thus, the response of the pancreas to secretin shows a sharp decrease in the first few days of ligation. A similar type of curve was obtained for the hourly amylase output in the juice. In the rabbits ligated for 4 days, the amylase output was only 16 per cent of the control.

Histological study of the pancreatic tissue (fig. 3, *a-d*) revealed that after 12 hours of ligation, the large ducts became dilated and there were protoplasmic changes with pale staining and vacuolization in some of the acinar cells. After 24 hours of ligation, some of the intralobular ducts also became dilated (fig. 3*b*). Two days after ligation, a great deal of the parenchyma was degenerated and there was an infiltration of fibrous tissue. The number of ductules was increased. The lobules showed atrophy and some of the degenerated acinar cells were replaced by the dilated ductules (fig. 3*c*). This picture was more clear in the 4-day ligated pancreas in which more acinar cells had undergone degeneration and more ductules had become visible and more dilated. The epithelial cells of the intralobular ducts became flat (fig. 3*d*).

As can be seen from figure 1, both the volume and the enzyme output of the juice showed a slow and gradual decrease from the low value attained at 4 days to zero by the end of two weeks. It is interesting to note that in the scanty pancreatic juice obtained from the animal 2 weeks after ligation, there was no amylase at all. The corresponding histological sections from the animals during this period showed that a gradually progressive degenerative process was taking place in the acinar cells. The infiltration of the fibrous tissue and the round cells became more pronounced as the ligation time increased and finally at the end of 2 weeks almost all of the acinar cells were gone (fig. 3, *e-g*). The loss of acinar cells was associated with hyperplasia and enlargement of the ductules. This resulted in a few dilated ductules surrounded

by infiltrated fibrous tissue remaining in each of the lobules as seen in figure 3g. Usually the gradual replacement of the acinar cells by the dilated ductules after a longer period of ligation was associated with an atrophy of the lobules.

A further study of the above-mentioned functional changes was carried out by measuring the response of the pancreas to a supramaximal dose of secretin and of pancreozymin. As shown in figure 2, the same type of curves was obtained as in figure

Fig. 1. EFFECT OF LIGATION of pancreatic duct upon the action of constant secretin administration on the volume and amylase output of pancreatic secretion (25 rabbits).

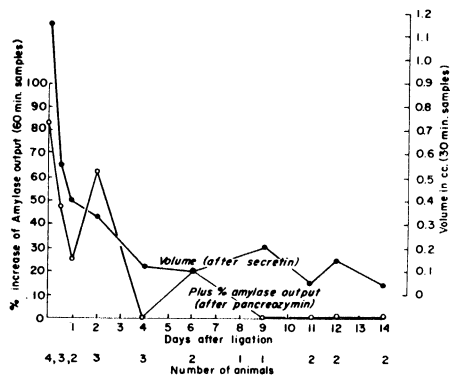
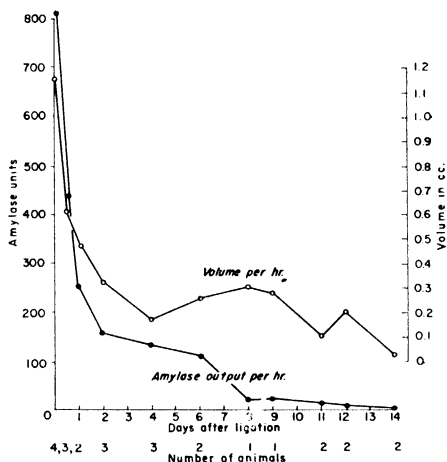


Fig. 2. EFFECTS OF LIGATION of pancreatic duct upon the action of a single supramaximal secretin dose and upon the action of pancreozymin (25 rabbits).

1. The 0.5-mg. dose of the highly purified secretin was believed to be a supramaximal dose in rabbits and the same was true of a 10-mg. dose of pancreozymin. The volume of juice collected within 30 minutes after the secretin injection and the percentage increase after pancreozymin in amylase output as compared to the basal level served as measurements. As can be seen from figure 2, the volume curve showed a sharp drop up to the 4th day of ligation and after that a gradual decrease takes place. In the curve showing the increased percentage of amylase output after pancreozymin, the general similarity to the amylase output curve of figure 1 is evident.

The complete disappearance of the increased enzyme response to pancreozymin started on the 9th day of ligation. It is noteworthy that the effect of pancreozymin

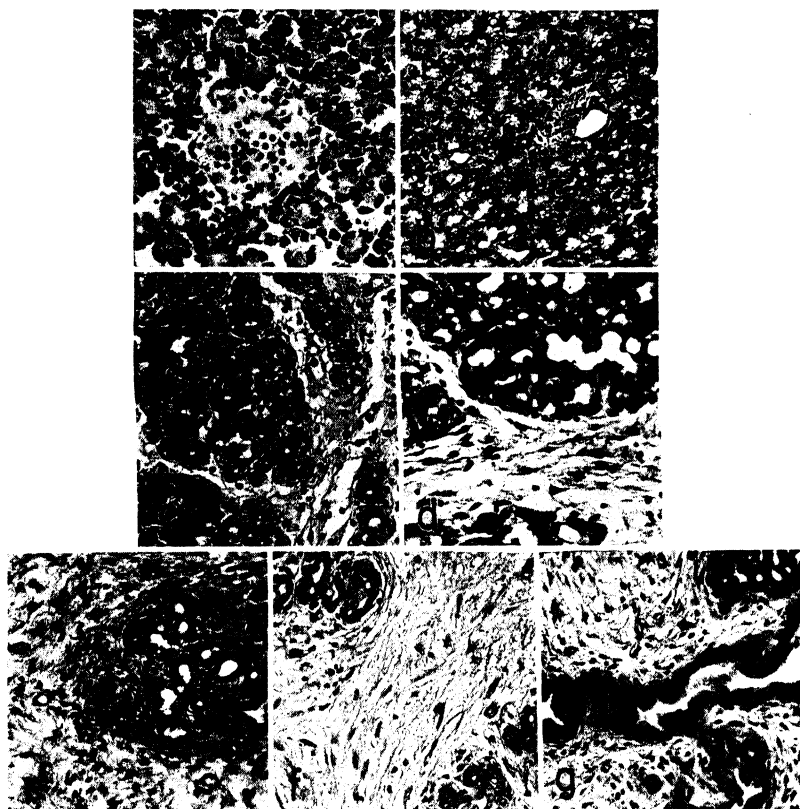


Fig. 3. PANCREAS OF RABBITS, varying lengths of time after ligation of the duct. H. & E. stain. $\times 150$. *a*: Control, no ligation, normal acinar and islet tissue. *b*: After ligation for one day. Some of the acinar cells have vacuoles and stain palely. A few intralobular ducts show dilatation. *c*: After ligation for 2 days. An increase in the number of ductules and a decrease in acinar cells is seen. The islands of Langerhans are normal. Fibrous connective tissue in the interlobular spaces is increased. *d*: After ligation for 4 days. Ductules are increased in number and dilated, with flattening of the lining epithelium. Most of the acinar cells have disappeared. *e*: After ligation for 6 days. The lobules are reduced in size and number. Heavy infiltration with fibrous and fatty tissue has taken place between the lobules. The islands of Langerhans appear normal. *f*: After ligation for 11 days. Further decrease in size of lobules. They now contain only a few dilated ductules and are surrounded by fibrous tissue. All the acinar cells are gone. *g*: After ligation for 14 days. All that is now left is a few dilated intralobular ducts and the moderately dilated interlobular ducts, surrounded by fibrous and fatty tissue.

tends to disappear in the degenerating pancreas before the disappearance of secretin action.

It has been shown repeatedly that alkaline phosphatase occurs only in the cells lining the intralobular ductules of the pancreas and is absent from the acinar cells (10, 5) (fig. 4*a*). In order to add assurance histologically that it was the epithelial

cells of the dilated ductules which were left and not the acinar cells, especially at the later stages of degeneration, a histochemical method of staining the enzyme alkaline phosphatase was used.

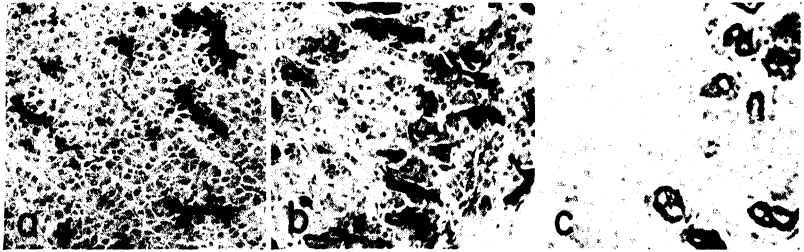


Fig. 4. PANCREATIC TISSUE from rabbits at various lengths of time after ligation of the pancreatic ducts. Gomori's alkaline phosphatase technique. $\times 150$. *a*: Normal control. The epithelial cells of the ductules and the centroacinar cells are positive for alkaline phosphatase but the acinar cells are negative. *b*: Intensification of the positive reaction in the ductule cells 2 days after duct ligation. The acinar cells remain negative. *c*: Fourteen days after ligation of the duct the reaction is still strongly positive in the few remaining dilated ductule cells.

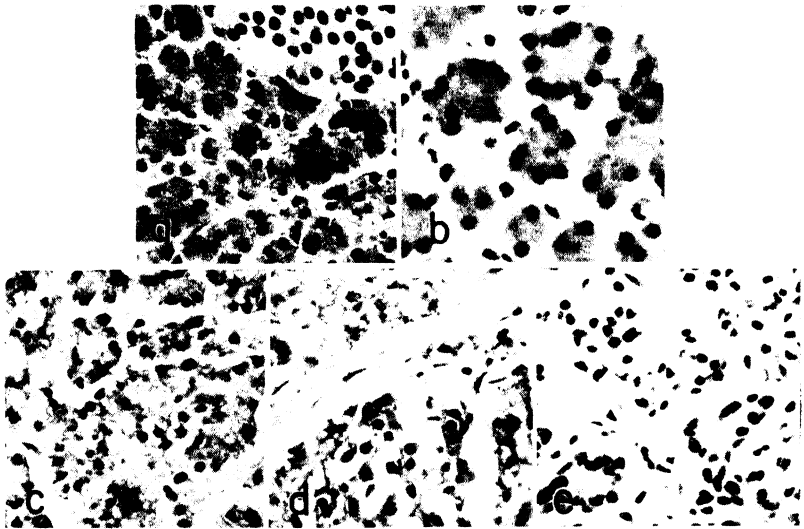


Fig. 5. PANCREATIC TISSUE from rabbits at various time intervals after ligation of the duct. Gomori's lipase method. Counterstained with hematoxylin. $\times 150$. *a*: Normal control. The acinar cells are filled with coarse lipase active granules. The dark color of the nuclei in this and *b*, *c*, *d*, and *e* photomicrographs is due to the counterstain. *b*: Twelve hours after ligation. The acinar cells are still positive for lipase. *c*: One day after ligation. Decrease in lipase-active granules. *d*: Two days after ligation. Further decrease in lipase staining. *e*: Four days after ligation. Lipase granules are entirely absent.

It was shown that within 12 hours of ligation, the phosphatase in the ductule cells showed no change except that in some of the infiltrated fibrous tissue the reaction becomes positive. However, the enzyme began to increase after 2 days of ligation.

tion at which time the ductules became dilated and their number increased (fig. 4*b*). At the end of 2 weeks, only the phosphatase-positive lining cells of the dilated ductules were left in the atrophic lobules indicating the entire absence of the acinar cells. The infiltrated fibrous tissue showed only a slightly positive phosphatase reaction (fig. 4*c*).

In order to show histologically the relation between the length of the period of ligation and the appearance of zymogen granules, a histochemical method of staining the enzyme lipase was adopted (see fig. 5). Within 12 hours of ligation, the lipase granules in the acinar cells showed no visible quantitative change as compared with those of the control animals (fig. 5*a*). However, from 12 hours on, a progressive disappearance of lipase granules in the pancreas was evident (fig. 5*b* and *c*) and after 2 days of ligation, the lipase granules were greatly reduced and only very few scattered ones are seen in the parenchyma (fig. 5*d*). From the 4th day on, the lipase reaction began to make its disappearance entirely (fig. 5*e*).

DISCUSSION

We had hoped that this study might give some information on the question of the separate function of the acinar cells and the centroacinar and duct cells. Although the tendency was in the direction which would support the hypothesis (11) that the former secrete only enzymes whereas the latter are responsible for most of the water secretion, the differences in the degree of depression of these two functions were not great enough to warrant an unequivocal conclusion.

Obstruction is believed to play an important role in the pathogenesis of pancreatitis (12). The present study permits an evaluation of the pancreatic changes produced by obstruction alone, without the additional factors of excessive stimulation of secretion and vascular impairment which are required to cooperate with obstruction if edema and necrosis of the gland are to occur.

In clinical practice when secretin is used to test pancreatic function it is usually found that the volume and bicarbonate show abnormally low values in early cases of chronic pancreatitis and only at a later stage is the enzyme output also reduced (13). This suggests that the type of damage occurring in these clinical cases is different from that produced by obstruction of the ducts in the experimental animal. When pancreozymin becomes available for clinical use, it may help to clarify this apparent dissociation of function.

SUMMARY

The effect of ligation of the pancreatic duct upon the action of secretin and pancreozymin in rabbits has been observed and a correlated histological study of the pancreas has been performed. Ligation of the pancreatic duct brought about a marked change in the pancreas both histologically and functionally. The rate of decline in functional performance was very rapid in the first 4 days of ligation and then fell gradually so that by the end of 2 weeks no pancreatic secretory activity was present. The loss of function after different durations of ligation was correlated closely with the picture of degeneration of the cells as revealed histologically.

The observations indicate that the effect of pancreozymin tends to disappear in the degenerating pancreas before the disappearance of secretin action.

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EFFECT OF TEMPERATURE UPON SURVIVAL OF THE EVISCERATE RAT

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IN THIS study eviscerate rats survived for the longest periods of time when the temperature of the surrounding air was 26°C. As the temperature was increased, the survival time was decreased.

METHODS

Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. The two-stage procedure of evisceration has been described (1). When the animals reached a weight of 250 ± 2 gm., they were anesthetized (intraperitoneal injection of 18 mg. of cyclopentenyl-allyl-barbituric acid sodium) and were subjected to the second stage of evisceration. Asepsis was preserved in both stages of the operation. Intravenous injections of solutions containing 0.9 per cent sodium chloride and varying concentrations of glucose (C. P. Dextrose, Merck) with regular insulin (Lilly) in the amount of 4 units/24 hours/rat were made by a continuous injection machine which delivered fluid from each of 6 syringes at the rate of 20 cc. in 24 hours. The glucose load is expressed as milligrams of glucose per 100 grams of rat per hour (mg/100 gm/hr.).

Infusions were made into the saphenous vein of the right hind leg and were started within 5 minutes following removal of the liver. The animals were secured in a supine position on an animal board and were enclosed in a cabinet which permitted the regulation of temperature within 0.5°C. The time of survival was determined by the use of a heart-beat amplifier (Model A, Upjohn) which was designed to amplify the D.C. potential generated by the heart beat and to actuate a 6-point recording mechanism (Leeds and Northrop Micromax S). This apparatus gives a permanent visual record of an all-or-none response of the amplifier to the beating heart for 6 animals simultaneously.

EXPERIMENTS AND RESULTS

In *experiment 1* (fig. 1) all of the rats were given a glucose load of 44 mg/100 gm/hr. with insulin. Six rats were tested at each of the following temperatures: 24°, 26°, 28°, 30°, 32°, 34°, 36°, and 38°C. The average survival time was greater at 26° than at 24° and as temperature was increased the survival times were decreased so that at 38° the average survival was less than 10 per cent of the average survival at 26°. Since the glucose requirement of the eviscerate rat is increased by a rise in temperature, it can be assumed that a glucose load of 44 mg/100 gm/hr., adequate for the maintenance of the blood glucose level at 26°, fails to prevent hypoglycemia at higher temperatures. Hypoglycemia was probably a factor limiting the survival of the rats at the higher temperatures.

In *experiment 2* (fig. 1), 8 groups of 6 rats each were given glucose loads which were known from previous study (2) to prevent hypoglycemia during the first three hours. The tolerance for glucose decreased with time. The glucose loads in relation to temperature were as follows: 70 mg/100 gm/hr. at 24°, 70 mg/100 gm/hr. at 26°; 80 mg/100 gm/hr. at 28°, 90 mg/100 gm/hr. at 30°, 100 mg/100 gm/hr. at 32°, 130 mg/100 gm/hr. at 34°, 140 mg/100 gm/hr. at 36°, and 150 mg/100 gm/hr. at 38°. The average survival was greater at 26° than at 24° and as the temperature was increased the average survival decreased. Although the survival times at the higher temperatures were improved over the values of *experiment 1*, the survivals at 24°, 26° and 28° were less than for the corresponding temperatures in *experiment 1*. The rats on the higher glucose loads at these temperatures developed hyperglycemia and

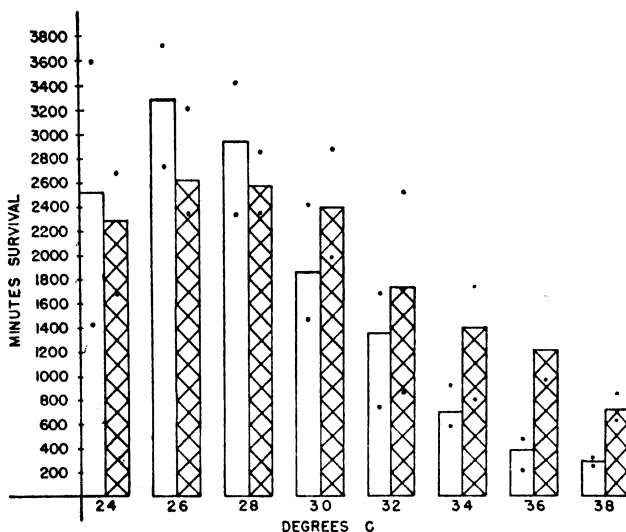


Fig. 1. EFFECT of temperature upon time of survival. □ = glucose load 44 mg/100 gm/hr. ▨ = variable glucose load.

glycosuria due to the fall in glucose tolerance which occurs during prolonged survivals so that an additional limiting factor becomes operative.

DISCUSSION

These data represent an addition to the great volume of evidence that temperature is a factor of major importance in many, if not all, problems involving quantitative measurements in biology. The principle should be recognized that the temperature-response relationship is an important dimension of any metabolic problem and that temperature should either be under control or its relationship to response explored.

We have carried on an amicable debate with Dr. J. A. Russell, Department of Physiological Chemistry, Yale University, regarding the optimal temperature for

metabolic studies on the eviscerate rat. A temperature of 26.5° has been used as standard in the laboratory because this temperature is optimal for survival and it has been desirable to extend certain studies for periods of 24 and 48 hours. The body temperature of the eviscerate rat falls rapidly to levels approaching that of the surrounding air. Dr. Russell has quite properly emphasized that at the abnormally low body temperatures of our experiments, metabolic processes are slowed and it is hypothetically possible for certain normally occurring enzymatic reactions to drop out at the lowered temperature. For these reasons the studies on eviscerate rats in the Yale laboratories require the artificial warming of the animal to sustain a normal body temperature. We believe that warming the skin to sustain the deeper tissues of the rat at normal temperatures introduces a new and undesirable factor. The surface of the skin of the rat is normally at a much lower temperature than the interior of the body. Apparently this gradient is required for normality since the intact, unanesthetized rat may die when exposed to room temperatures of 38° for several hours. The rat can neither sweat nor pant. This laboratory animal, so resistant to most forms of stress, is much more sensitive to heat than man. Russell and Capiello (3) state that eviscerate rats kept at room temperature frequently shivered and in many cases may have been in some degree of shock. Our animals usually shiver during the first two or three hours following evisceration but not thereafter. We have not found evidence of shock until the animals approached death. Our eviscerate rats are capable of showing vigorous response to the faradic stimulation of muscle over a period of several hours, a behavior incompatible with a state of circulatory collapse. Although we may not have reached agreement with the Yale group as to the optimal temperature for metabolic studies on the eviscerate rat, we are all fully agreed that changes in temperature have important effects upon the rates of metabolic processes and that it must be kept under control.

SUMMARY

Male rats were given continuous injections of glucose and insulin following evisceration. The times of survival were determined at 24° , 26° , 28° , 30° , 32° , 34° , 36° and 38°C . Eviscerate rats survived for the longest periods of time when the temperature of the surrounding air was 26°C . As the temperature was increased the survival time was decreased.

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OXYGEN TRANSPORT AND UTILIZATION IN DOGS AT LOW BODY TEMPERATURES

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WHEN a mammal hibernates its body temperature may fall to as low as 4°C. One of the earliest forms of general hypothermia to be investigated for its effect upon oxygen consumption was that associated with hibernation (1-6). In this state it has been found that the amount of oxygen consumed falls with increasing depth of hibernation but varies with the activity of the animal. These animals seldom shiver but observations are apparently difficult to obtain without disturbing their slumber. Benedict (5) in an excellent study reports oxygen consumption of only 3 per cent to 10 per cent of normal in deep hibernation.

Similar studies have been conducted upon non-hibernating mammals exposed to cold. Oxygen consumption in humans has been measured at temperatures as low as 28° C. (7-11) and in dogs, rats and rabbits at 19° C. (12-15). Reports of the effect of lowered body temperature upon oxygen consumption have been somewhat varied. Values ranging from a 300 per cent increase to 50 per cent decrease have been observed. One investigator (16) reported an oxygen consumption of zero for 1.7 hours with survival in an infant rat exposed to low temperature. These investigators realize that the high values for oxygen consumption are due to shivering invoked by the cold. The control of shivering with anesthetic agents and the effect of cold itself both tend to depress the respiratory center and thus introduce a further source of error in the form of developing hypoxemia.

In the present paper studies on dogs at induced low temperatures are reported. The oxygen consumption is studied with a view to correlating observed values with oxygen requirements of the tissues in hypothermia.

METHODS

Mongrel dogs of medium size after fasting for 20 hours were close-clipped and cooled by being placed in a controlled temperature room at an arbitrary level of -6 to -12°C. Occasionally an anteroom at 0°C. to 5°C. was used in the later stages to retard the rate of cooling and facilitate observations. Anesthesia was used to induce body cooling and to prevent shivering and increased muscle tonus. Barbiturates, curare and ether and either alone or in various combinations were tried. Two and one-half per cent secenal administered intravenously was finally selected as the anesthetic of choice. The initial doses of barbiturate were all administered on a proportional weight basis. Supplementary anesthetic during cooling to control the shivering varied somewhat in amount from animal to animal.

During the process of cooling, frequent observations were made on the respira-

tory and heart rate, rectal temperature, conscious level, and reflexes. In the majority of the experiments continuous visual observations and periodic recordings were made on a cathode-ray electrocardiograph.¹ This proved a great help in determining the condition of the animal for, without the electrocardiograph in the preliminary experiments, it was often difficult at low body temperatures to determine whether the animals were alive or dead because of the greatly weakened pulse and absence of respiratory movements. Indeed several authors writing on this subject have mentioned this difficulty.

To determine oxygen consumption two methods were used. In the first experiments the expired air was collected in a Douglass bag over a known period. Gas volumes were measured by a water gasometer and gas analyses for oxygen and carbon dioxide were carried out with a manometric Van Slyke apparatus (17). This procedure

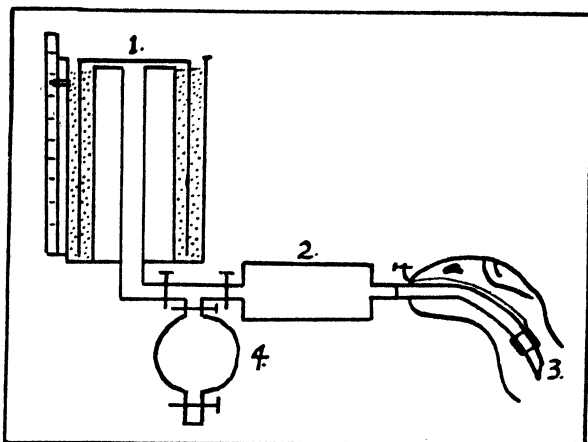


Fig. 1. SCHEMATIC DIAGRAM of spirometer circuit. 1. Bell-type spirometer. 2. Carbon dioxide absorber. 3. Intratracheal catheter with inflatable cuff. 4. Oxygen administration bag.

yielded the respiratory quotient and a figure for oxygen consumption. In the later experiments the dogs were connected by means of an air-tight endotracheal catheter to a one-liter spirometer filled with oxygen. Artificial respiration was carried out by intermittent manual pressure on the spirometer bell during a test period and maintained between tests by switching to an anesthetic bag connected with this system as shown in the diagram (fig. 1).

Samples of arterial blood for determination of oxygen saturation and carbon dioxide content were taken concurrently as the temperature fell. These samples were collected under oil and analyses were carried out on the manometric Van Slyke apparatus (18). The values obtained were not corrected for the increased amount of dissolved gas present at the lower temperatures. To obtain valid samples, in view of

¹ Smith and Stone Limited, electrocardiograph manufacturers, Georgetown, Ontario. Supplied by the Division of Aviation Medical Research, R.C.A.F.

the intense peripheral vasoconstriction and stasis existing at low temperatures, mixed venous blood was taken from the right auricle by a catheter inserted through the jugular vein. Arterial samples were withdrawn from a catheter passed up the femoral artery to the aorta. Both catheters were inserted under anesthesia and their position usually checked by x-ray before cooling was commenced. The lumen of the catheter was filled with a measured amount of hypertonic saline and heparin, sufficient to fill the catheter system.

Body temperatures were measured by a thermometer inserted 10 to 12 centimeters into the rectum. In a few experiments these readings were compared with coincident copper constantan thermal junction temperature readings using a Brown recorder (table 1). Since the temperature in the rectum and the right auricle never

TABLE 1. COMPARISON OF MERCURIAL RECTAL THERMOMETER AND COPPER-CONSTANTAN THERMOCOUPLE RECORDINGS

THERMOMETER °C.	THERMOCOUPLE °C.		
	Rectum	Heart	Muscle
27.5	27.5	26.5	19.0
25.8	25.5	25.0	17.5
24.5	24.7	24.0	16.5
23.0	23.0	22.0	
25.0	25.5		24.0
20.0	21.0	20.0	19.0
20.0	21.0	20.0	18.5
18.5	19.0	19.0	17.0
31.0	31.0	30.5	
29.8	29.8	29.2	26.0
26.5	26.8	26.5	22.2
16.0	16.0	15.6	13.2
18.8	18.8	17.8	13.0

differed by more than 1°C., it was concluded that body temperature values as read from a rectal thermometer would be satisfactory.

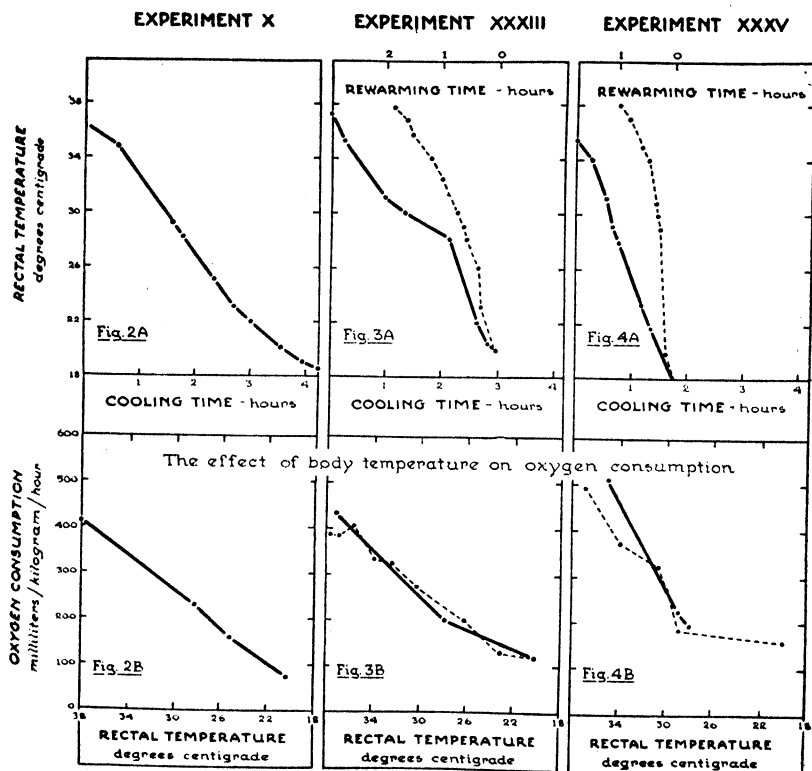
OBSERVATIONS

Experiments were carried out on 27 dogs. On exposure to cold air under the conditions outlined, the body temperature fell at an average rate of 6°C. per hour as illustrated graphically (figs. 2A-7A) in 6 representative dogs. It can be seen from the graphs that the rate of cooling varied somewhat probably because of variations in size and age of the dogs as well as the occasional use of the previously mentioned anteroom. Variation in depth of anesthesia which would ordinarily be an important factor was controlled as much as possible by using the minimum amount of anesthetic necessary to control muscle tremor.

In general with proper control of shivering each dog showed a progressive fall in body temperature, heart rate and respiratory rate. When the rectal temperature

had fallen to about 28°C., cold narcosis supervened, an anesthetic agent was no longer necessary to maintain relaxation, and a little later spontaneous respirations ceased. In the dogs that were not revived heart action usually ceased at 22°C. to 18°C.

As can be seen from the graphs, the rate of rewarming was faster than the rate of cooling. The heart rate increased rapidly and respirations appeared at approximately 27°C. Shivering was not a factor in rewarming as long as the animal was sub-

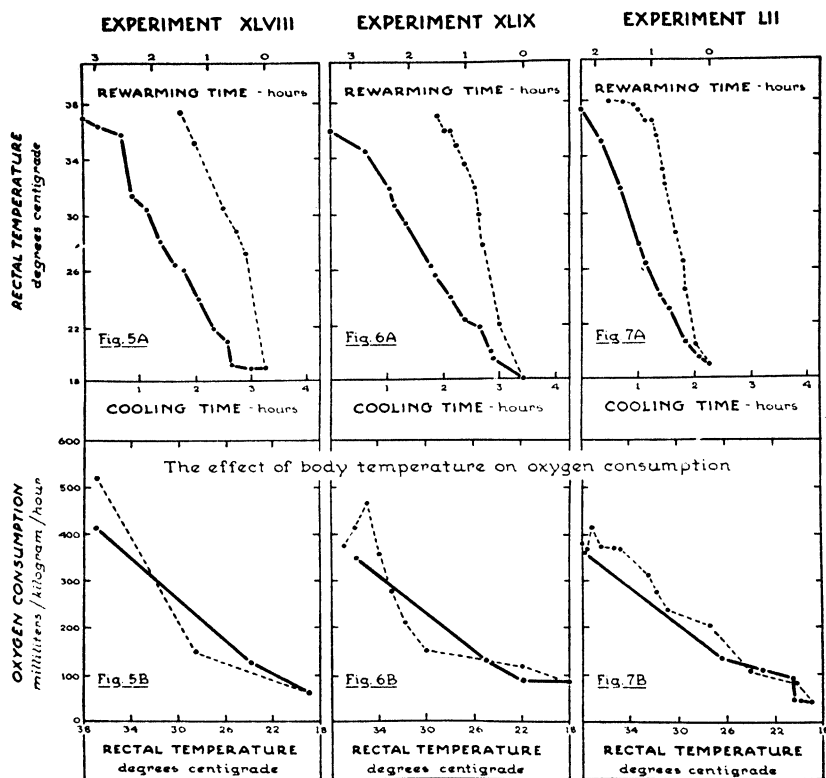


Figs. 2-4. SOLID LINE represents readings during cooling and dotted line during immediate rewarming.

merged in warm water. The later stages of rewarming were associated with tachycardia and hyperventilation. Table 2 summarizes the protocol of a representative dog showing the method of observation and cooling.

One hundred twenty determinations of oxygen consumption at various body temperature levels both during cooling and rewarming were made on animals who were neither shivering nor hypoxemic. The relationship between body temperature and oxygen consumption in individual dogs is shown in table 2 and illustrated graphically in figures 2B to 7B. The values for all dogs studied are plotted as a scatter graph (fig. 8), which shows the same general reduction in oxygen consumption with

fall in rectal temperature. In all these graphs the relationship is almost linear. Extrapolation would suggest that at body temperatures around 10° to 12°C . the oxygen consumption would be minimal, if not nil, suggesting a possible state of suspended animation. Unfortunately it is not possible with our present methods to maintain life in the dog at these temperature levels, much less revive him.



Figs. 5-7. SOLID LINE represents readings during cooling and dotted line during rewarming. 5B. Immediate rewarming. 6B and 7B. Rewarming after being maintained at a body temperature of 18° - 19° for 0.5 hours and 4.3 hours, respectively.

SIGNIFICANCE OF VALUES FOR OXYGEN CONSUMPTION

In attempting to obtain a true index of tissue oxygen consumption in general hypothermia, we were faced with four important questions: 1) How important is it to control all shivering? 2) Is the respiratory mechanism in this method of cooling adequate to maintain full oxygen saturation of arterial blood during cooling? 3) Is the circulatory system adequate to supply the tissue oxygen requirements? 4) Are the values for oxygen consumption a true index of tissue requirement, or does an oxygen deficit occur in the tissues?

It is obvious that the occurrence of shivering, a failure in the respiratory or the circulatory mechanisms, or the presence of an oxygen deficit could alter the results and render the conclusions invalid.

1) *How important is it to control shivering?* The control of shivering in the early stages of cooling was a major problem. At the same temperature level oxygen consumption was increased if shivering occurred. This was evident even with the minor degrees of shivering represented by a fine tremor. Thus careful control of this factor during the process of cooling was necessary. Table 3 indicates the effect

TABLE 2. METHOD OF OBSERVATION AND COOLING

RECTAL TEMP.	TIME	ROOM TEMP.	SPONTANEOUS RESPIRATION	PULSE	ARTERIAL OXYGEN SATURATION	OXYGEN CONSUMPTION	OXYGEN CONSUMPTION	REMARKS
°C.	hr.	°C.	breaths/min.	beats/min.	vol. %	ml/kg/hr.	% of 'Normal'	
38.0	0.0	21.1	16	136	98.9	410	100	'Normal' oxygen consumption measured under anesthesia. Artificial respiration.
28.0	3.05	-6.0	8	118	100.0	229	55.8	No anesthetic agent necessary. Breathing room air intermittent with oxygen by artificial respiration.
25.0	3.40	-6.0	5	80		159	38.8	Artificial respiration with oxygen.
20.0	4.50	0.0	0	40	100.0	75	18.2	Artificial respiration with pure oxygen at 12/min.
18.0	5.45	0.0	0	16	98.5			Dog was actually cooled to 16°C. at which level heart became completely irregular and death ensued.

Experiment. 15.2 kg. male dog. Anesthetized with 15.2 ml. of 2.5% secenal and intubated. Ecg. connected, rectal thermometer inserted. Ether-oxygen mixture administered to obliterate increased muscle tonus and minor tremors. 'Normal' oxygen consumption measured using a bell spirometer. Animal moved to cold room (-6.0°C).

observed in 5 dogs at a selected body temperature (28°C.). Again, figure 9 provides a comparison of the oxygen consumption values in the presence and absence of shivering.

2) *Is the respiratory mechanism in this method of cooling adequate to maintain full oxygen saturation of arterial blood during cooling?* In our first experiment, the dogs became cyanosed. If the animal was becoming hypoxemic during cooling, the figures obtained for oxygen intake would be lower than the actual tissue oxygen consumption. With the early repression of respiration by anesthesia and cold it was found that the arterial oxygen saturation did fall unless artificial respiration was carried out using oxygen.

Using the method of collecting expired air in a Douglas bag it was not possible to carry on artificial respiration and supplement oxygen. When this technique was used the animals became definitely hypoxemic and their oxygen consumption, calculated from an analysis of the expired air, was much lower than that of the subsequent animals in which the arterial oxygen saturation was maintained by artificial respiration. This difference increased as the hypoxemia became more severe.

The reduction in oxygen saturation which takes place on cooling without artificial respiration or added oxygen was observed in 4 dogs. Arterial oxygen saturation as low as 58 per cent was recorded. An abnormally low figure for oxygen consumption was a constant finding when the arterial blood was becoming desaturated. Table 4 and figure 9 illustrate this point. The arterial oxygen saturation in *experiment 3* returned to normal during the process of revival.

By supplying adequate oxygen and using artificial respiration the arterial oxygen saturation can be maintained at normal or around 95 per cent saturation with oxygen during the process of cooling and survival. This is illustrated during the cooling stage in table 2. Blood analyses of this type were carried out in 10 dogs of our series, and showed that this technique regularly kept the oxygen saturation normal.

3) *Is the circulatory system adequate to supply the tissue oxygen demands?* Having recognized that artificial respiration and oxygen were required to maintain a normal oxygen saturation in the arterial blood, the next problem was to determine whether the circulation was adequate with the reduced blood pressure and cardiac rate and increased circulation time observed at lower temperatures. It was felt that the degree of reduction in oxygen content in the blood after passing through the vascular bed would serve as an index of adequacy of oxygen supply to tissues. Simultaneous arterial and mixed venous blood samples of 4 dogs undergoing cooling were analyzed for their oxygen content. The results were examined for evidence of abnormal reduction of oxygen content in the venous blood, i.e. increased arterio-venous oxygen differences.

Table 5 shows comparative figures in a representative dog at various tempera-

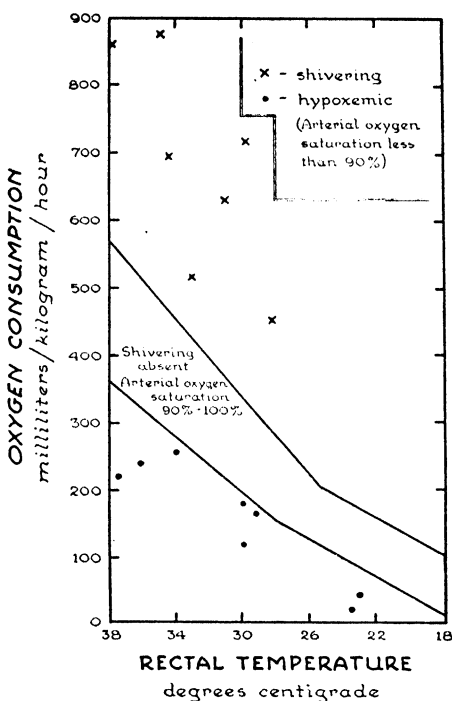


Fig. 8. SCATTER GRAPH of all oxygen consumption findings during cooling. Shivering was controlled and arterial oxygen saturation was maintained at 90% to 100% in these experiments.

ture levels. Carbon dioxide contents were in keeping with oxygen change. It will be seen that in spite of reduced circulation during cooling no abnormal fall in oxygen content occurred during passage of blood through the tissues. It is concluded that at least down to 18°C. in the presence of satisfactory heart action the circulation was adequate during cooling. It is possible that at lower temperature levels the circulation may not be adequate to supply tissue oxygen demands.

TABLE 3. EFFECT OF SHIVERING ON OXYGEN CONSUMPTION AT BODY TEMPERATURE 28°C

<i>Experiment</i>	SHIVERING CONTROLLED				SLIGHT SHIVERING	MARKED SHIVERING
	10	35	33	Average	5	33
Oxygen consumption ml/kg/hr.	229	199	201	209	445	713

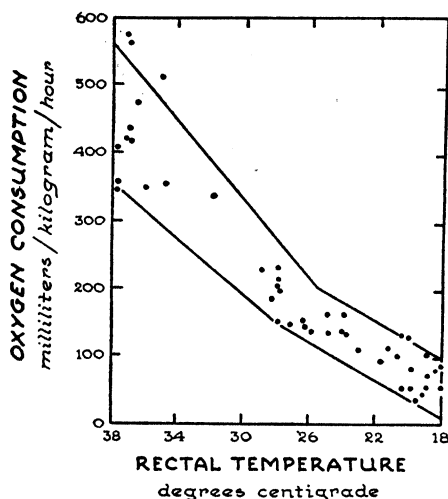


Fig. 9. GRAPHIC ILLUSTRATION of effects of shivering and developing hypoxemia upon oxygen consumption. This graph is drawn to the same scale as figure 8. The lined area of figure 9 is identical with the zone outlined in figure 8.

4) Are the values for oxygen consumption a true index of tissue requirements or does an oxygen deficit occur in the tissues? Having established what appears to be satisfactory respiratory and circulatory function the key problem of utilization of oxygen by the tissues at reduced temperature arises. Do the cold and the associated biochemical change in the blood produce an oxygen deficit in the tissues in spite of apparently adequate oxygenation? The oxygen dissociation curve is influenced by many factors (19) and in this study presumably by temperature and pH predominately. Lowered temperature tends to shift the curve to the left, thus for a given oxygen tension in the tissues the blood oxygen becomes less available in the hypothermic state.

The opposite effect is produced by an increase in acidity or carbon dioxide content which will shift the curve to the right and increase availability of oxygen to the tissues. One would suspect that the increase in carbon dioxide content of blood in

hibernating animals (20-22) must be a factor in aiding the reduction of oxy-hemoglobin in the hypothermic state.

In the early experiments it was found that the carbon dioxide content of arterial blood was increased in animals that were cooled without artificial respiration or oxygen except in very rapid cooling. When oxygen was given with carbon dioxide ab-

TABLE 4. ERROR IN USING OXYGEN INTAKE TO MEASURE OXYGEN CONSUMPTION IN THE PRESENCE OF A FALLING OXYGEN SATURATION

<i>Experiment 3</i> Douglas bag method. No artificial respiration.			<i>Experiment 10</i> Spirometer method. Artificial respiration and supplementary oxygen.		
BODY TEMP.	ARTERIAL OXYGEN SATURATION	OXYGEN CONSUMPTION	OXYGEN CONSUMPTION	ARTERIAL OXYGEN SATURATION	BODY TEMP.
°C.	vol. %	ml/kg/hr.	ml/kg/hr.	vol. %	°C.
37.5	90.0	378	410	98.9	38.0
30.0	94.0	137	229	100.0	28.0
23.0	75.0	35	159	100.0	25.0

TABLE 5. ARTERIO-VEINUS OXYGEN DIFFERENCES IN THE COLD STATE

TEMPERATURE	OXYGEN CONTENT		ARTERIO-VEINUS OXYGEN DIFFERENCE
	Arterial	Venous	
°C.	vol. %		vol. %
33.0	22.5	18.5	4.0
26.0	22.9	19.8	3.1
18.0	23.0	19.5	3.5

TABLE 6. EFFECT OF CARBOGEN ON VENOUS BLOOD CARBON DIOXIDE CONTENT AT TEMPERATURE RANGE 25°C.-20°C.

CARBON DIOXIDE CONTENT—VOL. % (MEAN VALUES)			
Body temperature 38°C. (normal)		Body temperature 25°C.-20°C. (undergoing cooling)	
Room air Spontaneous respiration (11 exper.)	Room air Spontaneous respiration (4 exper.)	Oxygen and carbon dioxide absorber Controlled respiration (4 exper.)	Carbogen Controlled respiration (5 exper.)
48.7	56.5	40.5	51.2

sorber, the arterial oxygen saturation was maintained but the carbon dioxide content fell to a level below the normal. The use of Carbogen, 5 per cent carbon dioxide, 95 per cent oxygen mixture, however, was found to maintain both the arterial oxygen and carbon dioxide content during cooling and revival. This has been used since the improvement in the survival rate. Mean values for carbon dioxide content of arterial blood are given in table 6.

The problems of tissue oxygen deficit were considered from three aspects:

a) *General late effects of hypothermia.* It was felt that an index of tissue hypoxia during cooling might be found by the observation of delayed effects on the animal

after survival such as mental retardation, general ill health, anemia, etc. It was realized that such detrimental effects could be due to the direct effect of cold as well as hypoxia and that gross observations of this kind on an animal such as a dog would not be acceptable in application to humans. Smith (23) has followed a group of cancer patients who had been treated by general hypothermia and found no evidence of mental change. In 50 autopsies on previously treated cases he saw no suggestion of previous anoxia in brain sections.

In this study note was made of general deportment and response of 11 animals which survived after cooling. These were gross observations and a more detailed study of late effects is being conducted. Of the 11 survivals examined over a period of 3 to 7 months after cooling, 10 dogs appeared normal. The 11th dog appeared somewhat emaciated, with some mange of face and a fall in weight from 8.15 kg. to 7.2 kg. 6

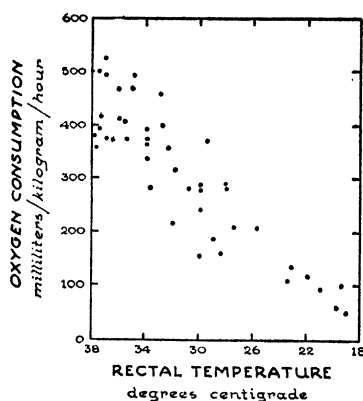


Fig. 10. SCATTER GRAPH of all oxygen consumption findings during rewarmings.

weeks after cooling. Before killing this dog, blood examination revealed W.B.C. 8,120; R.B.C. 4,200,000; Sedimentation Rate 2 mm. (Westergren).

b) *Oxygen utilization on rewarming.* It was felt that any oxygen deficit occurring during cooling might be recognized by recording oxygen consumption during revival with a search for evidence of increased oxygen demand out of proportion to the body temperature. Figures 3 to 7 inclusive indicate the increasing oxygen consumption with return to normal temperature.

These graphs roughly parallel the changing oxygen consumption on cooling and show no evidence of a sudden great demand for oxygen once the bonds of reduced temperature are lifted. As the animal's body temperature rose to normal the oxygen consumption often increased slightly above the parallel line for cooling for a short period. This phase of increased oxygen consumption was coincident with one of hyper-ventilation, and may be attributed to the increased muscular work of overbreathing and thus was not the result of tissue oxygen deficit. By the time normal body temperature was reached the oxygen consumption had settled down to approximately the normal precooling level.

Figure 10 is a composite graph showing the relationship between a rising body temperature and the oxygen consumption, when shivering is absent and the blood

oxygen saturation is normal. It is of interest to compare this graph with figure 8 and note the close relationship which exists.

These observations indicate that an oxygen deficit does not exist in the tissues of animals cooled in this manner and that the figures obtained for oxygen consumption give a true index of the level of tissue metabolism at the temperature levels studied.

c) *Does a prolonged state of hypothermia produce oxygen deficit in the tissue?* It is possible that the experiments in which the animals were immediately revived after cooling did not allow sufficient time for tissue oxygen deficit to occur. To test this hypothesis dogs were cooled to 19° and maintained at the temperature for periods varying from 0.5 to 4.3 hours during which time repeated oxygen consumption determinations were made. Some of the observations made on two of these dogs are represented graphically in figures 6 and 7, but the long period of hypothermia is not illustrated. The oxygen consumption did not change during the relatively constant state of hypothermia.

Two of the 3 animals were revived and their oxygen uptake was measured during the period of rewarming. These showed a rise in oxygen consumption which paralleled the fall noted in the cooling graphs. In both of these a rise was noted between 30° and 37°C., indicated in figure 6*B* and figure 7*B*, which was associated with temporary hyperventilation similar to that seen with immediate revivals and associated with temporary hyperventilation. It is to be noted that within a few minutes after the dogs had resumed a temperature of 37°C. their oxygen consumption had returned to a level similar to that of the precooling state.

It was concluded that hypothermia at temperatures down to 19°C. and maintained at this low level for as long as 4.3 hours does not produce an oxygen deficit in the tissues.

DISCUSSION

This study indicates that there is a steady fall in the oxygen consumption of animals as the body temperature is reduced to low levels. The observations are limited to temperatures ranging from normal (38°C.) down to 18°C. Life could not be maintained at lower levels. The close relationship between temperature and oxygen consumption in different dogs is significant in view of the diversity of size, age and breed.

The variation in the results of previous workers was noted in the brief survey of the literature. Most students of metabolism of hibernating animals agree that there is a steady decrease in oxygen consumption on entering this state. Shivering in such animals is apparently not a great feature, although voluntary movement is. Rasmussen (20, 21) and Dubois (22) have shown a greater than normal arterial oxygen saturation of blood in the hibernating animal and suggest that this indicates oxygen storage and that at least part of the oxygen absorbed has not been used in cell metabolism.

The observations in the literature which indicate an increased consumption of oxygen in non-hibernating mammals as the body temperature is lowered have been made for the most part in the temperature range down to 25°C. only and it is in this zone that shivering if uncontrolled is often a feature. In humans Smith (7), Geiger (8) and Vaughan (11) show that the majority of their values at reduced temperatures are below normal, findings which are consistent with the fact that their patients were relatively quiet. Their values for oxygen consumption registered below normal and

taken at a relatively fixed body temperature still show a wide variation. The variations may be due to differences in respiratory effort as influenced directly by cold or by depth of anesthesia. In deep anesthesia there may be a fall in arterial oxygen saturation, which, as we have shown, will give low values for oxygen consumption (fig. 9).

Working with humans, Dill and Forbes (9) reported an increase in oxygen consumption in the majority of their cases of hypothermia. This undoubtedly is attributable to gross shivering and voluntary movement, as noted in their protocols.

Working with dogs, Gross-Brockhoff and Schoedel (12) showed a consistent initial increase in oxygen consumption above normal which they attributed to shivering and which fell to varying degrees as cooling continued and shivering diminished. In animals under heavy anesthesia the initial increase was minimized and oxygen consumption was progressively reduced to subnormal levels. They noted oxygen desaturation only as a terminal event in their most heavily anesthetized animals. It would appear that oxygen desaturation was not a problem in their experiments, presumably due to the fact that their dogs were maintained at a higher conscious level.

In the present study it is felt that the causes of variation in oxygen consumption have been minimized by the preventing of shivering and by maintaining full oxygen saturation of the arterial blood at all times both during cooling and rewarming. Two possible sources of error however exist. First, the necessary use of anesthetics may of itself alter oxygen consumption; second, observations made at any given point while the animal was being gradually cooled or warmed might not represent satisfactorily the average oxygen consumption of the tissues.

The use of anesthesia during the induction of cooling was found necessary not only to obtain relaxation and to gain a true measure of oxygen consumption, but to eliminate the protective shivering and facilitate rapid cooling of the animal. An anesthetic was sought which would control shivering and which would be quickly eliminated once the period of shivering had passed. This would give as nearly a pure state of cold narcosis as possible. Seconal, as used, satisfied these requirements.

Covett (24) has shown that sodium pentobarbital had no direct effect on the oxygen consumption of dogs whose body temperature is maintained at a constant level. We did not attempt to study the effect of anesthesia per se, but did try to maintain a constant level of anesthesia in all our experiments.

Our observations of oxygen consumption were made while the animal's body temperature was falling at an average rate of 1°C. per 10 minutes. Two animals only were stabilized at 19° to 20°C. for 1.5 and 4.3 hours. Repeated observations showed no significant change in oxygen consumption during this period.

Some bacteria and fish can survive temperatures near absolute zero (-273°C.). Poikilotherms, or cold-blooded animals, as highly developed as the frog can exist for months frozen solid and apparently using no oxygen. The tendency of a premature baby to assume its environmental temperature thus necessitating an incubator and the increased resistance to cold found in our studies in young dogs (25) both suggest some retained poikilothermic characteristics, in the development of non-hibernating forms. What anatomic or physiologic change has taken place in the evolution from hibernating to non-hibernating forms, however, is not yet known.

SUMMARY

Hypothermia to 18°C. was induced in dogs by exposure to cold air. They were anesthetized sufficiently to control all shivering and increased muscle tonus. Full arterial blood oxygen saturation was maintained by artificial respiration when necessary. Measurements of oxygen consumption were taken during cooling and rewarming. Oxygen consumption fell consistently with reduction in body temperature down to 18°C. and rose in proportion during rewarming in a way that was parallel. Evidence is presented that under the conditions of these experiments a tissue oxygen deficit did not develop during the period of cooling.

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INTERRELATIONSHIP OF THE CARDIO-RESPIRATORY EVENTS IN ANOXIA¹

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IT is recognized that breathing is controlled by many stimuli: chemical, proprioceptive, cardiovascular and cortical (1-5). Although a few investigators have become temporarily lost in the cul-de-sac of unit chemical control of respiration, most have quickly backed out when they realized that even if some chemical did regulate most of the pulmonary ventilation, it could not explain the cortical control of breathing. Furthermore, the most important respiratory stimulus, work, cannot be shown, in spite of tortuous efforts, e.g. Bernthal (3), to exert its effects by a chemical pathway.

CONTROL OF BREATHING

There are undoubtedly many factors influencing breathing. As Haldane and Priestley (2) phrase it: "If we attempt to determine, one by one, the 'factors' in the regulation of breathing, the sum of the supposed factors turns out to be illusory, since no one of them is a constant quantity. The evaluation of each factor depends on its varying relation to the others."

The control of breathing may be compared with a complex extension of the newer computing machines. Both mechanisms probably have the negative feed-back and reverberating circuit patterns described by Northrop (6), Wiener (7) and others. With respect to the reflex controls of breathing, their detailed operation can easily be considered as a complex of binary switching devices. On the other hand, with respect to the chemical controls of breathing, they probably do not act on such a simple 'on-off' system. Rather they follow the type of mechanism found in computing machines of the differential analyzer type. The scale of values in both chemical controls of breathing and in the differential analyzers is continuous. In both mechanisms, each increment of change in one factor affects all the other factors. The resulting changes in breathing therefore can be considered as an additive total of many on-off switching devices, in combination with the pattern evolving from the interaction of many continuous-scale mechanisms.

Ultimately the patterns of breathing will probably be described and predicted by the simultaneous solution of many differential equations. This demands the use of complex computing machinery. As a first approximation to the computing machine approach, we have elected to investigate the interaction of a portion of the controlling mechanisms. For analysis of complex functions several statistical methods have proved very fruitful, as for example, multiple correlation analysis, or the analysis of mental abilities by Thurstone's (8) multiple factor technique. The complex control of breathing lends itself well to this type of statistical treatment, for we can not only estimate the comparative weights of the various stimuli but also, given the actual data, determine how well we can predict and therefore how complete is our knowledge of the function under investigation.

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Although physiologists have long been aware that the respiration is controlled by several stimuli, the first formal attempt to put the several parts together and to account for breathing as the net result of several stimuli was recently made by Gray (5, 9, 10). Utilizing the Henderson-Hasselbalch formula and published data on the relation of the breathing to oxygen tension and carbon dioxide tension, he deduced the equation:

$$VR = 0.22H + 0.262 p\text{CO}_2 - 18 + \frac{105}{10^{0.088p\text{O}_2}}$$

where VR is the ratio of actual alveolar ventilation to resting alveolar ventilation, H is the hydron concentration times 10^9 and p represents blood tension of the respective gases. This formula applies to "any condition (in the steady state) where only chemical agents are influencing ventilation" (9). It specifically excludes conditions in which, say, proprioceptors or the cortex are influencing respiration.

This bold attempt has been adversely criticized (11), but the fact remains that it was used by Gray to predict with accuracy breathing in various states like prolonged inhalation of CO_2 , chronic pulmonary emphysema and uncomplicated low pressure anoxia (9). It has also enabled Gray to partial out the effects of the three main chemical stimuli on respiration, showing that now one and now another, predominates in respiratory control.

We have recently completed an experiment measuring the terminal physiological events in unanesthetized dogs during various kinds of overwhelming accident: breathing N_2 , breathing 2.43 per cent O_2 in N_2 , breathing one per cent CO , rebreathing through soda lime, obstructive asphyxia, fresh and salt water drowning, and exsanguination (12). These data are especially appropriate for a multiple factor analysis of breathing, since we measured simultaneously with the breathing, the blood O_2 , CO_2 , lactic acid, acidity, and arterial and venous blood pressures. Gray's formula should not be applied to our data for several reasons: first, the dogs were for the most part strongly anoxic, with blood O_2 much lower than in the medium types of anoxia explored by Gray; second, they were not in a 'steady state' (i.e. the ventilation well adjusted to moderate degrees of anoxia or acidity or $p\text{CO}_2$) as required in Gray's formulation; and third, no measurements were made of O_2 -tension, but only of O_2 -content and saturation. In spite of these differences, as a matter of curiosity we have applied Gray's formula to our data of dogs breathing N_2 , breathing 2.43 per cent O_2 in N_2 and rebreathing through soda lime. Only data from dogs, circulation of which was still well maintained, were used. As a first approximation to O_2 -tension, we used O_2 -saturation. Prediction of breathing by Gray's formula was fair for dogs which were undergoing anoxia from rebreathing, but it failed seriously in most conditions, as for example in the early apnea occurring in dogs breathing N_2 .

Gray included no estimate of the error of his prediction. It is evident that a process as labile as the breathing cannot be said to have been adequately described unless the analysis shows how great an error is involved in the prediction of its course. Such an estimate of the error in the predictions will also give us an index of the completeness of our present knowledge of the breathing in anoxia. Manifestly, another analysis of the multiple control of breathing over the whole range of anoxia is desirable. This will be attempted in the first portion of this paper.

METHODS

The methods used in studying overwhelming fatal anoxia (the term 'anoxia' meaning here oxygen deficiency of any degree, regardless of cause) have been des-

cribed in detail in three reports of the actual experiments (12). Briefly recapitulating, dogs under local but not general anesthesia were made to breathe N_2 or 2.43 per cent O_2 or to rebreathe through soda lime into a spirometer of 3.5-l. capacity. The pulmonary ventilation and respiratory rate were recorded with a spirometer; the systolic and diastolic arterial pressures, the venous pressure in the inferior vena cava and the intrathoracic pressure were recorded continuously by optical methods. At intervals during the course of the anoxia, arterial blood samples were obtained for analysis of O_2 , CO_2 , pH , lactic acid and densities of whole blood and plasma. From these measurements were deduced the following variables: the ventilation ratio following, in part, Gray's usage (9), is defined as the ratio of the observed ventilation to the ventilation of a dog of similar weight at rest. The arterial O_2 -saturation is the ratio of the observed O_2 -content to the product: Hb. content times 1.35. The plasma CO_2 -tension was derived from the whole blood CO_2 -content, the pH , the Hb. content, and the O_2 -saturation, employing the computational methods of Van Slyke and Sendroy (13). The observed pH , taken at room temperature, was corrected to the pH at 38° by Rosenthal's formula (14). The Hb. content was deduced from the blood and plasma densities by the formula of Phillips *et al.* (15).

Breathing pure N_2 caused death in 4 to 6 minutes. The last two conditions caused death in 9 to 21 minutes. We have excluded from this analysis any measurements taken when the animal was on the threshold of death, i.e. when the systolic pressure had fallen below 150 mm. Hg. The actual data studied are shown in the original reports (12).

Method of Analysis. Our analysis is based upon the following assumption: The accuracy with which we can predict a measurement from a knowledge of other measurements is an index of the extent of our knowledge of the factors controlling the predicted measurement. Any factor which does not add to the accuracy of the prediction has little influence on the variation of the predicted measurement.

We have selected the following measurements for study:

- VR: Ventilation Ratio, ratio of observed pulmonary ventilation at any given time to the ventilation of a dog of similar weight at rest.
- RR: Respiratory Rate, number of complete respiratory cycles per minute.
- pCO_2 : Carbon Dioxide tension, tension of gas in solution in plasma.
- Os: Oxygen Saturation, per cent saturation of arterial blood with O_2 .
- pH : Logarithm of the reciprocal of the hydron concentration.
- cH : Hydron concentration times 10^6 .
- Hb: Hemoglobin, grams of hemoglobin/100 ml. of whole blood.
- LA: Lactic Acid, milligrams of lactic acid/100 ml. of whole blood.
- VP: Venous Pressure, pressure (cm. H_2O) within the thoracic inferior vena cava.
- IP: Intrathoracic Pressure, pressure (cm. H_2O) within the pleural space.
- DBP: Diastolic Blood Pressure, the lowest pressure (mm. Hg) during the cardiac cycle as measured in the femoral artery.
- PP: Pulse Pressure, difference between systolic and diastolic pressure.
- HR: Heart Rate, number of complete cardiac cycles /minute.

From these measurements we have attempted to amplify Gray's concept of multiple controlling factors by deriving an empirical formula which would best predict pulmonary ventilation from a series of simultaneous measurements of other variables throughout a wide range of ventilations.

The simple linear correlation coefficients (r_0) of pulmonary ventilation to each of the 11 other variables were calculated and found to be those shown in table 1. The high correlation of VR and RR is due to the fact that they are recognized to be, in general terms, measurements of the same variant: the ventilation of the lungs.

TABLE 1. CORRELATION OF VENTILATION RATIOS WITH OTHER VARIABLES

VR to	pCO_2	O_2	pH	PP	HR	LA	DBP	VP	RR	Hb	IP
$r_0 =$	-.30	-.10	.31	.33	.25	-.04	.34	.02	.74	.02	.13

TABLE 2. CORRELATION (r_0) MATRIX

	VR	pH	pCO_2	DBP	O_2	VP	PP	HR	LA	RR	Hb
Ventilation Ratio (VR)											
pH		.31									
pCO_2		-.30	-.34								
Diastolic blood pressure (DBP)		.34	-.66	.00							
Oxygen saturation (O_2)		-.10	-.28	.65	.03						
Venous pressure (VP)		.02	.25	-.41	.10	-.54					
Pulse pressure (PP)		.33	.14	-.56	.00	-.38	.40				
Heart rate (HR)		.25	.10	.35	.34	-.34	-.08	-.69			
Lactic acid (LA)		.04	-.18	-.64	-.43	-.28	.28	.00	-.17		
Respiratory rate (RR)		.74	.18	-.02	.24	.26	-.11	.03	.22	-.24	
Hemoglobin (Hb)		.02	.28	-.24	.37	-.28	.15	.01	.05	-.10	.01
Intrathoracic pressure (IP)		.13	-.24	.21	.08	.09	.13	-.14	.18	-.08	-.02

TABLE 3. PARTIAL CORRELATION (r_1) OF VENTILATION RATIO WITH A SECOND VARIABLE, THE THIRD HELD CONSTANT (2.43% O_2 IN N_2)

	pH	Hb	pCO_2	O_2	LA	VP	IP	DBP	PP	HR
r_0	.58	.18	-.29	-.07	-.25	.18	-.34	.59	.26	.21
pH		.12	-.21	-.14	.09	.18	-.29	.23	.21	-.01
Hb			-.32	-.02	-.20	.14	-.35	.58	.30	.18
pCO_2				.01	-.45	.40	-.27	.61	.20	.16
O_2					-.29	.21	-.33	.61	.32	.21
LA						.23	-.32	.55	.18	.18
VP							-.41	.59	.22	.25
IP								.58	.27	.35
DBP									.13	.01
PP										.21
HR										

Because, however VR corresponds better to the metabolic needs of the animal, we shall rely on this measurement as the best gauge of the breathing. Diastolic blood pressure, pH , pCO_2 , PP , and HR all show the same order of relation to VR . If r_0 is defined as the index of determination, each of these measurements, considered

independently, determines about 10 per cent of the variance in ventilation. However, the algebraic sum of all these 5 factors does not account for 50 per cent of the variance. This follows because none of these factors is completely independent of any other as can be seen from table 2 where the complete r_0 matrix of correlations is given. An approximation of the interdependence of factors and hence the degree to which certain factors contribute to the combined variance was arrived at in a preliminary study of the first-order partial correlations (table 3) of the data from dogs breathing 2.43 per cent O_2 .

Table 3 shows that the pH , pCO_2 , VP and DBP have the most marked interdependence in VR correlations. In an attempt to amplify our knowledge of which combination of measurements would yield the highest accuracy in predicting VR , multiple correlations (R) were calculated but were not significantly greater than the simple r_0 correlations even out to third-order multiples. By reference to the data from all three types of anoxia, an attempt was then made to derive empirical formulas for VR , using the data from these measurements classically involved in the analysis of respiration. When only pH and pCO_2 are considered, the best prediction of VR is from the relations:

$$VR = 31.21 + 4.88 pH - 0.0976 pCO_2.$$

The correlation coefficient for this is 0.325 with a standard error of estimate of 3.5 VR units. When O_2 -saturation is included in the formula, the best prediction of VR is from the relation:

$$VR = 30.47 + 4.73 pH - 0.0255 pCO_2 - 0.0245 Os.$$

The correlation coefficient is actually decreased to 0.238 with a standard error of estimate of 3.09 VR units. These experimental calculations, and the outstanding lack of participation of O_2 -saturation in the variance of VR , emphasized the unreliability of simple correlation procedures.

All of these correlations are based upon an assumption that the best fit of 2 variables lies around a straight line. The linear correlation of VR to Os is $-.10$. Since anoxia, particularly in the dog, is a strong respiratory stimulus (at first), this figure is absurdly low. A scatter diagram (fig. 1) shows that a straight line is a very poor approximation to the data. But the application of lines of increasing mathematical complexity, the cubic equation, as drawn in figure 1 was found to be the line of best fit, the method of least squares being employed in its determination. The curvilinear correlation (ρ) of VR to Os is .74. Thus, if the linear assumption is not demanded, there is a high correlation between VR and Os . In the same manner the application of quadratic equations to cH , (used instead of pH since linearity is no longer required) and to pCO_2 result in correlation with VR of .64. These are significant increases over the simple linear correlations.

The square of the correlation coefficient is a measure of the percentage of variation in the dependent variable which could be said to be associated with the independent variable. Each of the factors, Os , pCO_2 and cH , when considered as acting independently, give correlations of .64 to .75; hence by squaring the coefficient they account for about 40 to 50 per cent of the changes in pulmonary ventilation. How-

ever, as in the case of the linear correlations, these cannot be algebraically summated since they are not mutually independent. The only way to estimate the combined effects of many variables on pulmonary ventilation is to calculate a series of multiple curvilinear correlations. A curvilinear correlation coefficient was calculated for each combination of variables by the least squares method. Because of the tremendous computational labor, no more than 10 simultaneous equations were used in any combination.

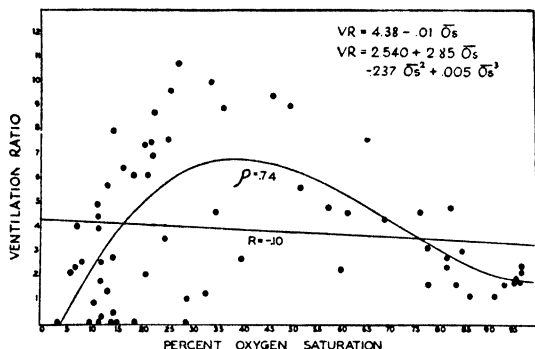


Fig. 1. RELATION OF BREATHING TO OXYGEN SATURATION

The diastolic blood pressure (*DBP*) and venous pressure (*VP*) were added to the analysis, since both of these are known to influence the breathing (2, 16). These 2 are maintained as shown in table 2, since they are good approximations in linear form. A series of the equations resulting for these solutions are as follows:

CORRELATION OF:	FORMULA
VR to <i>Os</i> :	$VR = 2.540 + 2.85 Os - 0.237 Os^2 + .005 Os^3$
VR to <i>cH</i> :	$VR = 6.989 - 0.413 cH + 0.009 cH^2$
VR to <i>pCO</i> ₂ :	$VR = 8.690 - 0.704 pCO_2 + 0.024 pCO_2^2$
VR to <i>DBP</i> :	$VR = 1.220 + 0.365 DBP$
VR to <i>VP</i> :	$VR = 4.01 + 0.042 VP$
VR to <i>Os</i> + <i>cH</i> :	$VR = 0.719 + 2.765 Os - 0.226 Os^2 + 0.005 Os^3 - 0.375 cH + 0.009 cH^2$
VR to <i>Os</i> + <i>cH</i> + <i>pCO</i> ₂ :	$VR = 0.744 + 2.497 Os - 0.190 Os^2 + 0.004 Os^3 - 0.353 cH + 0.008 cH^2 - 0.418 pCO_2 + 0.011 pCO_2^2$
VR to <i>Os</i> + <i>cH</i> + <i>pCO</i> ₂ + <i>DBP</i> :	$VR = 1.910 + 2.202 Os - 0.165 Os^2 + 0.004 Os^3 + 0.002 cH - 0.001 cH^2 - 0.293 pCO_2 + 0.001 pCO_2^2 + 0.315 DBP$
VR to <i>Os</i> + <i>cH</i> + <i>pCO</i> ₂ + <i>DBP</i> + <i>VP</i> :	$VR = -0.007 + 2.075 Os - 0.149 Os^2 + 0.003 Os^3 - 0.115 cH + 0.002 cH^2 - 0.326 pCO_2 + 0.004 pCO_2^2 + 0.219 DBP - 0.048 VP.$

The correlation coefficients (ρ) for an expanding series of these multiple curvilinear correlations are as follows:

VR to O_s74	VR to O_s + cH81
VR to cH64	VR to O_s + cH + pCO_282
VR to pCO_264	VR to O_s + cH + pCO_2 + DBP83
VR to DBP29	VR to O_s + cH + pCO_2 + DBP +	
VR to VP03	VP.....	.86

Thus, with one cubic, two quadratic, and two linear elements in the equation, about three fourths of the variance of pulmonary ventilation can be accounted for. The addition of further variables increases the computations to impractical extremes but a study of the correlation matrix in table 3 allows an estimate of the probable

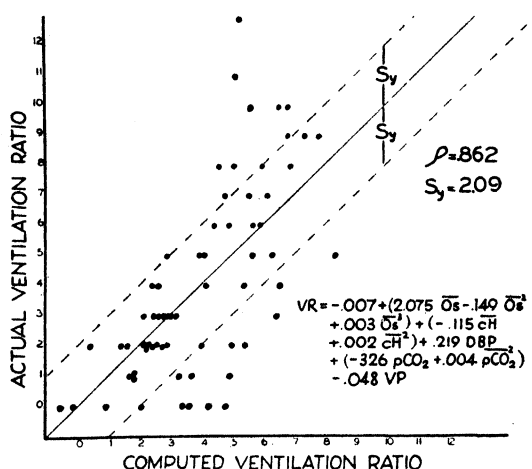


Fig. 2. PREDICTION OF BREATHING

course of such additions. By adding all 12 of the variables into the equation, the accuracy of prediction of VR may be increased slightly. However, because of the interdependence of each variable with each other variable and because we have not in our series of measurements completely covered all of the possible controlling factors which may influence VR, even the inclusion of all 12 variables into one prediction formula would not, it is felt, yield a perfect correlation.

The accuracy of the predicted VR, so far as we have gone, can be estimated by calculating the root mean square deviation of the estimated from the measured VR. This can be interpreted in much the same manner as a standard deviation. A scatter diagram to accompany such a computation from the formula including 5 variables is shown in figure 2. If each estimate of VR were 100 per cent accurate, all dots would fall along the straight line; but actually the root mean square deviation around this line is 2.09 VR units. Thus, if we know O_s , pCO_2 , cH , VP and DBP , we could predict to within two VR units in two thirds of the predictions. Since the total range of VR in these experiments was only 13 units, the predictions are not, in a physiological

sense, good. The obvious interpretation is that we have not measured, even indirectly, all of the factors controlling respiration.

A MULTIPLE FACTOR ANALYSIS OF ANOXIA

When the formulas for predicting pulmonary ventilation had been computed, a series of intercorrelations between each of the 12 measurements became evident. In order to develop a prediction formula, pulmonary ventilation was defined as a dependent variable determined by the interdependency of other variables. Similarly we could have developed a formula to predict any of the other variables.

In a sense, each of the 12 variables discussed is equally dependent or independent, depending upon the use to which the data are put. But there seems to be a pattern behind the 12 x 12 matrix of measurements, when all are considered equally depen-

TABLE 4. A NORMALIZED OBLIQUE FACTOR MATRIX ON THE DATA IN ANOXIA

	FACTOR A	FACTOR B	FACTOR C	FACTOR D
+1.00	$p\text{CO}_2$	$p\text{H}$		
+.90	O_s	Hb & DBP	VR	
+.80	HR			IP
+.70			RR	
+.60				VP
+.50		RR & VR		HR
+.40	RR		IP & HR	DBP
+.30			PP & O_s	
+.20				VR
All others less than $\pm .10$				
-.20				Hb
-.30		LA	Hb	
-.40				
-.50		IP		
-.60				
-.70				
-.80	VP			
-.90	LA & PP			
-1.00				

dent. Mathematical methods for determining such underlying patterns are complex and as yet incomplete. One method, the centroid multiple factor analysis technique of Thurstone (8), has shown excellent results in similar problems.

Method of Analysis. The method of Thurstone involves taking a matrix of correlation coefficients of a series of simultaneous measurements. This matrix is factored in a geometry of multi-dimensional space, and the axes are rotated to produce the least possible number of unique vectors from which one can reconstruct the original test matrix.

This process has been applied to the matrix shown in table 2. A resulting orthogonal factor matrix was put through 7 rotations to produce an oblique factor matrix. The test vectors were extended to unit length to produce the normalized factor matrix shown in table 4.

This chart may be interpreted much the same as Thurstone's factor analyses of psychological tests. Where, for example, many tests may have a loading with a verballity factor and hence to that extent measure the vector 'verballity', there may be one test which is unique in being loaded only with the verballity factor and hence may be called a pure test of verballity. With the use of this one test, the verballity of the subject is completely determined. Thus, each of our measurements has a loading in Factor A. However, *Os*, *HR*, *PP*, *LA* and *VP*, each highly loaded in Factor A, also have significant influence along at least one vector. Only $p\text{CO}_2$ is unique in Factor A; it has no influence upon the other factors. In the case of Factor B, only $p\text{H}$ is unique. These two factors can thus be named the $p\text{CO}_2$ and the $p\text{H}$ factors. Factors C and D are not completely determined by this set of data. In order to determine them a new set of measurements, involving other variables, is probably necessary.

Before interpreting the factor analysis, some cautions must be observed. The Thurstone method demands linear correlations. While *ch* was converted to $p\text{H}$ which yields good approximation to linearity, the $p\text{CO}_2$ correlations are better fitted to quadratic equations and some of the *O*₂ correlations are nonlinear. Hence the use of linear correlations in this method is bound to give faulty results. Further, the size of the sample limited the reference frame to a four-dimensional space. This is an artificial limitation, since one of the problems is to determine the number of dimensions, or factors, controlling the actions of the body during an anoxic accident. An even more serious criticism is that the type of measurements used was limited in scope. The inclusion of other equally important measurements, such as cortical control or proprioceptive activity, might completely change the picture presented in table 4.

However, some tentative results are shown by this introductory analysis. Factor A shows one measurement which is completely absent from all other factors: $p\text{CO}_2$ is completely loaded in Factor A and does not appear in any of the other factors. Factor B is dominated by $p\text{H}$, which does not appear in any other factor. $p\text{CO}_2$ and $p\text{H}$ thus define factors A and B, and may themselves be called prime factors of the matrix. Factors C and D are incompletely determined, but seem to relate themselves to pulmonary movements and blood pressures. There is no single measurement which can be called a prime factor for either of these two dimensions.

Probably the most important conclusion that can be reached from this trial analysis, and one which is not influenced by the faults in the data, results from the necessity of using an oblique reference frame. If the prime factors which appeared in the analysis were independent of each other, then these prime factors could be pictured in space on a right angle reference frame. Each prime factor could completely describe one dimension of the many-dimensional activities of the experimental animal and a separate effect of each prime factor could be determined. If $p\text{CO}_2$ or $p\text{H}$ could be considered 'prime', they could be said each to contribute a certain percentage to the control of the animal's life, and, by an extension of this principle, the effect of each on the breathing of the animal under any conditions could be determined. However, the right angle spatial dimensions do not yield an heuristic picture. In order to evolve a series of prime vectors it was necessary to use an oblique reference frame in which even the prime vectors are correlated with one another. Thus, the interpretation of these relationships must be very general. As in the case of the correlation analysis, none of these measurements is completely independent. There is

no evidence from our data that any of the changes that occur in any one of these peripheral measurements is responsible in itself for the series of events that follows. $p\text{CO}_2$ and $p\text{H}$, each primary vectors, are in themselves correlated in an oblique reference frame. The fact that an orthogonal reference frame could not be used emphasizes the interdependence of even the primary vectors.

In order for the multiple factor analysis technique to be valid, a method of dealing with curvilinear correlation will have to be found, or the experimental condition must be narrowed to a restricted range, as Gray (5) has done, wherein linear relationships are valid approximations. Also many other variables must be measured. It would appear from this pilot analysis that there are n factors contributing to the control of respiration. It is impossible to determine how many factors control the breathing except experimentally. It can be stated however that many more variables would have to be measured to get any specific number of prime factors.

It is interesting that the 2 vectors defined by the multiple factor analysis, $p\text{CO}_2$ and $p\text{H}$, have each been implicated in the literature as a single prime factor controlling respiration. Neither, as measured from the periphery of the body, can be so considered.

DISCUSSION

When Haldane and Priestley (2) wrote, as we have quoted at the start of this paper, that "The evaluation of each factor (in the regulation of breathing) depends on its varying relation to the others," they had in mind the sort of analysis of breathing that Gray (5) attempted and that we have attempted here. It is only by analyses like these that we will eventually be able quantitatively to state the relationships involved. For example, a statement, such as "The respiratory center is insensitive during anoxia," has little meaning unless we can say how sensitive it is to what stimuli at what degree of anoxia.

In our opinion, this attempt to formulate the respiratory behavior in anoxia has failed in the sense that the empirical equations derived show a considerable prediction error. But, in another sense, this is not a failure at all, because the error has been quantitated and thus we are shown how incomplete our knowledge is. The method would reveal similarly the degree of error in any other physiological variant studied; in fact, most of the generally accepted 'laws' of physiology, e.g. Starling's 'Law of the Heart,' hold only under certain specified, and usually rather narrow, conditions.

In Gray's formulation of the factors controlling breathing, the variation with the oxygen tension was found to be logarithmic; in our formulation, the variation was found to be cubic. As we have pointed out, the difference is a matter of range of anoxia studied; in Gray's study, normal and mild; in our case, normal, mild, moderate and severe. The normal and mild part of our curve could be, in fact, a logarithmic curve just as was Gray's. We have carried the analysis a step further than Gray and 'pushed the curve over the hump' into severe anoxia.

SUMMARY

An empirical formula predicting pulmonary ventilation in dogs in three types of anoxia has been developed, using O_2 -saturation, CO_2 -tension, $p\text{H}$, and diastolic

and venous blood pressures. The error involved (one root mean square deviation) in attempting a prediction of pulmonary ventilation from these measurements has been found to be about 2 in 13 ventilation units, one ventilation unit being defined as the dog's pulmonary ventilation at rest. It has been shown that the breathing in anoxia does not bear a linear relation to the O_2 -saturation, but rather that the breathing is best related to the O_2 -saturation by means of a cubic equation.

A multiple factor analysis of the dogs' physiological behavior in anoxia has also been made, resulting in further emphasis upon the mutual interdependence of all the measurements taken. The pH and the pCO_2 appear to be two of many prime factors in the dogs' over-all reactions to anoxia.

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CORONARY BLOOD FLOW AND CARDIAC OXYGEN CONSUMPTION IN UNANESTHETIZED DOGS¹

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THE nitrous oxide method for the determination of blood flow was introduced by Kety and Schmidt (1) in 1945 and applied by these workers to the measurement of cerebral blood flow. Goodale (2), Eckenhoﬀ (3), and others combined this method with coronary sinus catheterization to determine coronary blood flow in the anesthetized dog. The nitrous oxide method was first used in man by Bing and co-workers in the study of normal, diseased and failing hearts (4). The present experiments were begun in order to study cardiac oxygen consumption and efficiency in experimental mitral insufficiency in dogs. During the progress of this study a considerable discrepancy was found between the results obtained in unanesthetized dogs and those previously reported in anesthetized animals (5). It seemed, therefore, of interest to reinvestigate coronary blood flow and cardiac oxygen consumption in the unanesthetized dog because of the importance of establishing normal values as well as demonstrating the validity of the nitrous oxide method.

MATERIAL AND METHODS

Mongrel dogs varying in weight from 9 to 20 kg. were used. The animals were trained to lie quietly on a table with a Blalock dog mask in place (6).

At the onset of the experiment the animal was placed on a fluoroscopic table. The external jugular vein in the neck and the saphenous artery in the leg were exposed. Clean but not aseptic technique was used throughout. No anesthesia other than the local injection of 2 per cent procaine was given. A modified no. 7 or 8 Courmand catheter was inserted into the jugular vein (7), and the animal was turned into the left lateral position. This position rather than the left anterior oblique position recommended by Goodale (2) was used because the landmarks in the region of the coronary sinus ostium could be visualized more sharply. In the lateral position under the fluoroscope the coronary sinus ostium is usually located in the region where the anterior shadow of the inferior vena cava merges with that of the heart. The entrance of the catheter into the coronary sinus could easily be recognized by observing the sharp bend made by the tip of the catheter. The catheter was inserted to a depth of not more than 3 cm. (2). Further confirmation of the fact that the coronary sinus had been entered was obtained by noting the dark color of the blood obtained. A slow drip of heparinized saline solution prevented clotting of blood in the catheter.

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The depth to which the catheter is inserted into the coronary sinus is of importance. If the tip lies barely within the ostium, the sample obtained will be a mixture of right auricular and coronary sinus blood. This possibility was carefully investigated by Goodale, and is not thought to be a great hazard (2). In only one instance in about 50 experiments did the high oxygen content of the blood obtained

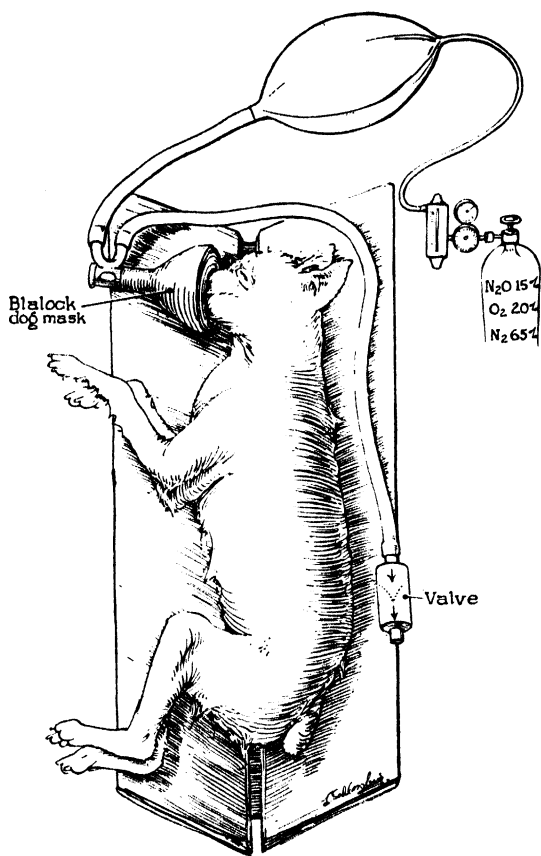


Fig. 1

lead to the suspicion that contamination with right auricular blood had occurred. On the other hand, if the catheter is too deeply inserted into the sinus, the possibility of obstruction of the sinus arises. The stasis produced by such obstruction would tend to considerably decrease the coronary flow. A further hazard of too deep insertion of the catheter is the possibility of withdrawing blood directly from coronary capillaries instead of from coronary veins. In one experiment in a small dog obstruction was thought to have been present, for the venous curve approached the arterial curve very slowly, and the flow was about 25 per cent of that obtained in other experiments. Goodale has reported the finding of thrombosis of the great cardiac vein after experiments in which the catheter was deeply inserted and left in place several hours (2).

In all except the earliest experiments a second catheter was inserted through the same vein into the right ventricle or pulmonary artery for the collection of mixed venous blood. This made possible the simultaneous determination of coronary blood flow and cardiac output. For the collection of arterial samples the saphenous artery was cannulated with small-bore polyethylene tubing. The catheter in the coronary sinus and the arterial tubing were connected to manifolds as suggested by Kety and Schmidt (8). The nitrous oxide desaturation method as suggested by Goodale (9) and used in man by Bing (4) was used in preference to the original saturation method of Kety and

Schmidt (8). Using the desaturation method the possibility of leaks around the dog mask is avoided. Saturation of the animal with nitrous oxide was accomplished by allowing the animal to breathe a mixture of 15 per cent nitrous oxide, 20 per cent oxygen, and 65 per cent nitrogen for 8 to 10 minutes. This has been found sufficient time to saturate heart muscle completely with nitrous oxide. The respiratory apparatus is shown in figure 1.

At the end of the saturation period arterial and venous samples were obtained for the determination of nitrous oxide concentration at the saturation level. The mask was then removed and the animal allowed to breathe room air. During the first 4 minutes of desaturation arterial and coronary venous blood samples were collected through the manifolds simultaneously, in immediate succession, and at a uniform rate over a one-minute period. A total of 8 samples were obtained. For various reasons continuous sampling during the desaturation period was used rather than intermittent sampling as described in the original method by Kety and Schmidt (1). Respiratory fluctuations are of no consequence, a more accurate measurement of the total arteriovenous difference is obtained, and calculation of the blood flow is simplified. During the 4-minute desaturation period a mixed venous sample was taken from the catheter lying in the right ventricle or pulmonary artery. Oxygen consumption was measured by the closed-circuit method on a Benedict-Roth spirometer. Mean femoral arterial pressure was obtained with a mercury manometer. Primary healing of the skin incisions was usually obtained by approximating the skin edges with silk sutures and locally injecting 50,000 units of penicillin. Experiments on the same animal were separated by an interval of 24 hours or longer. A transfusion equal in volume to the blood removed was usually given at the end of the test.

Blood was collected in syringes which had been lightly oiled with mineral oil. A minimal amount of oil was used, for nitrous oxide is very soluble in this material. Five drops of one per cent heparin solution were added to each syringe to prevent clotting. The samples were analyzed for oxygen and nitrous oxygen content on the manometric apparatus of Van Slyke and Neill (10). The method of Kety and Schmidt for oxygen analysis in the presence of nitrous oxide was used (5). In the nitrous oxide analysis certain modifications have been found helpful: 1) the oxygen absorber was freshly prepared, extracted, and analyzed in duplicate for the presence of absorbed gas; 2) the volume of distilled water introduced into the measuring cup of the Van Slyke apparatus was measured accurately.

CALCULATIONS

The coronary blood flow is calculated from curves representing the changing concentration of nitrous oxide with time in arterial and coronary venous blood (1). The presence of an experimental or analytical error is indicated when smooth curves are not formed, or when the venous curve does not approach the arterial in a uniform fashion (1). As coronary sinus blood is considered to be chiefly blood draining the left ventricular muscle (11), the blood flow obtained represents left ventricular flow. It should be emphasized that the coronary blood flow obtained by the nitrous oxide method expresses the flow of blood per unit of left ventricular tissue (expressed as cc/100 gm/min.) (4). In order to obtain the blood flow for the entire left ventricle,

the blood flow per 100 gm. must be multiplied by the left ventricular weight. The oxygen consumption per 100 gm. of left ventricular muscle is the product of the blood flow per 100 gm. and the coronary arteriovenous difference (the oxygen extraction). The weight of the left ventricle in the intact animal was estimated by using the tables obtained by Herrman (12). This investigator found that the left ventricular weight comprises a constant fraction of the body weight (average value of 0.0037). In the animals that were killed the left ventricle was weighed and the actual ventricular weight substituted for the estimated ventricular weight. The degree of correlation between estimated and actual ventricular weight was good. In only one instance was the difference between estimated and actual heart weight over 6 grams. Sixty-five per cent of the ventricular weights were measured at autopsy, and 35 per cent were estimated by the formula of Herrman.

The cardiac output was calculated by means of the Fick principle (13). Surface area was calculated from the formula, $SA = \text{weight (kg.)}^{2/3}$. Cardiac work was expressed in kilogram meters per minute. It was calculated from the formula of Starling: work (kg. meters/min. = cardiac output (cc/min.) \times mean aortic pressure (cm. Hg) \times 13.6 (14). No allowance was made in this calculation for the velocity energy, since at rest this component represents less than 10 per cent of left ventricular work (15). To express the oxygen consumption of the left ventricle in the same units as left ventricular work, the oxygen consumption was multiplied by the work equivalent of oxygen at a respiratory quotient of 0.82 (cc. oxygen = 2.059 kg. meters) (16).

Cardiac efficiency for any portion of the heart is an expression of the relation of the energy consumed to the work performed. As coronary sinus blood consists of blood which has perfused left ventricular muscle, the work-energy cost relationship must be calculated for the left ventricle only. The following equation was used:

Mechanical efficiency of left ventricle (percentage)

$$= \frac{\text{work of total left ventricle (kg. meters/min.)}}{\text{energy cost of total left ventricle (kg. meters/min.)}} \times 100.$$

As the nitrous oxide method is essentially a variation of the Fick principle, it is subject to the same limitations. Hence, with high flows the total arteriovenous difference will be small, and a small analytical error will alter the result considerably. With flows greater than 200 cc/min./100 gm., this error is quite appreciable, but only two of the experiments reported here had flows exceeding this value.

RESULTS

Twenty-seven experiments were performed on a total of 14 animals. Table 1 shows the oxygen content of blood from the coronary sinus, the femoral artery, and the right ventricle, and the arteriovenous oxygen difference between peripheral arterial and coronary sinus blood, and between peripheral arterial and mixed venous blood respectively. It may be seen that the oxygen content of the coronary sinus blood ranged from 2.22 to 7.26 vol. per cent. The coronary arteriovenous oxygen difference (the left ventricular oxygen extraction) had a mean value of 11.9 vol. per cent with a standard deviation of 1.05. The arteriovenous oxygen difference between

peripheral arterial and mixed venous blood varied from 3 to 6 vol. per cent. The difference in oxygen content of coronary sinus blood and right ventricular blood was

TABLE 1

ANIMAL NO.	DATE	WEIGHT	SURFACE AREA BODY SURFACE	O ₂ CONTENT CORONARY SINUS	O ₂ CONTENT RIGHT VENT.	O ₂ CONTENT FEMORAL ARTERY	A-V O ₂ DIFFERENCE	
				vol. %	vol. %	vol. %	CORONARY F.A.—C.S.	SYSTEMIC F.A.—R.V.
		kg.	m ²				vol. %	vol. %
1	3/21	18	.77	5.34	13.24	17.80	12.46	4.56
1	3/22	18	.77	5.04	10.45	16.47	11.43	6.02
1	3/28	18	.77	4.64	11.74	15.30	10.66	3.56
2	3/11	14	.65	5.31	13.54	19.09	13.78	5.55
2	3/14	14	.65	4.30	9.95	16.61	12.31	6.66
3	2/1	19	.80	4.25	12.43	17.25	13.00	4.82
3	2/11	19	.80	2.80	10.76	15.80	13.00	5.06
4	1/31	14	.65	4.13	13.02	18.23	14.10	5.21
4 ¹	2/2	14	.65	3.58	17.28	19.28	15.70	2.00
5	3/1	9	.49	3.76	9.59	15.16	11.40	5.57
5	3/8	9	.49	3.44	7.89	13.10	9.66	5.21
6	1/29	12	.58	3.10	7.26	13.20	10.10	5.94
7	3/10	9	.49	2.90	8.79	14.44	11.54	5.65
8	3/2	11	.55	3.57	7.96	13.87	10.30	5.91
8	3/17	11	.55	2.22	9.10	14.48	12.26	5.38
9	4/5	20	.83	6.48	12.28	18.60	12.12	6.32
9	4/7	20	.83	3.93	10.26	15.62	11.69	5.36
10	4/6	16	.71	5.77	14.32	18.30	12.53	3.98
10	4/8	16	.71	4.20	10.78	14.90	10.70	4.12
11	5/6	16	.71	5.94	13.40	18.00	12.06	4.60
11	5/9	16	.71	7.26	13.06	18.56	11.30	5.50
12	4/21	16.5	.73	3.97	11.33	16.60	12.63	5.27
12	4/26	16.5	.73	4.36		16.73	12.37	
12	4/27	16.5	.73	3.07	9.30	14.90	11.83	5.60
13	5/23	16.5	.73	4.85	15.81	20.18	15.33	4.37
14 ¹	5/26	14.5	.66	3.12	14.86	18.19	15.07	3.33
14	5/27	14.5	.66	6.34	13.34	18.31	11.97	4.97

¹ Not considered a basal animal.

marked (table 1). The high oxygen extraction by left ventricular muscle, and the marked difference in oxygen content between coronary sinus blood and mixed venous

blood, are in agreement with findings reported by Harrison (17) and by Eckenhoff (5) in dogs and by Bing in man (4).

Table 2 illustrates the data obtained on blood flow, oxygen consumption, work and efficiency of left ventricular muscle. The left ventricular blood flow/100 gm. varied from 79 to 220 cc/minute, with a mean of 133 and a standard deviation of 35. These figures are much higher and also more variable than those previously reported for anesthetized dogs, using a similar technique (3, 5). The variability of blood flow per unit weight of left ventricle is probably related to differences in total left ventricular weight. This subject will be discussed in detail in a subsequent paragraph.

The left ventricular oxygen consumption per 100 gm. ranged from 9.58 to 24.04 cc/minute, with a mean of 15.75 and a standard deviation of 3.54 (table 2). These values are much higher than those reported in the anesthetized animal (5) or in normal man (4). Since the values for the arteriovenous oxygen difference between arterial and coronary venous blood showed little variation, the wide range of values for oxygen consumption per 100 gm. was the result of the great variation in ventricular blood flow. It will be shown in a subsequent paragraph that as a result of the higher values for left ventricular oxygen consumption, the calculated ventricular efficiencies were much lower than those reported in anesthetized dogs (5). The efficiencies were, however, quite similar to those reported in normal man (table 2) (4).

In contradistinction to the wide variation in left ventricular blood flow per 100 gm., the blood flow calculated for the total left ventricle showed a much smaller standard deviation (13.7 as compared to 35). Table 2 shows that the figures for total left ventricular blood flow ranged from 52 to 103 cc/minute, with a mean of 70.1. As mentioned above, it is probable that the variations in blood flow per unit weight were the result of differences in total ventricular weight. Comparable data on the coronary blood flow of the entire left ventricle of dogs are not available in the literature.

The oxygen consumption of the entire left ventricle ranged from 6.04 to 15.18 cc/minute, with a mean of 8.52. The standard deviation was 2.22, whereas the standard deviation for oxygen consumption/100 gm. was 3.5. Consequently, there was considerably less scatter than was present in the figures obtained for the oxygen consumption per unit of heart weight. It is probable that here again the discrepancy between values per unit of weight as compared to values for the entire ventricle is the result of variations in ventricular weight. The figures for left ventricular oxygen consumption shown in table 2 are similar to those found in normal man (4).

The cardiac output varied from 2.8 to 6.7 liters per square meter of body surface per minute (table 2). These values are higher than those reported by Marshall (18), by Blalock (19) and by Wiggers (20), but agree with those reported by Eckenhoff in anesthetized (5) and by Bing in unanesthetized (21) dogs respectively. It is probable that the use of anesthesia is responsible for some of the differences reported (22). The mean femoral blood pressure showed little variation from a mean value of 120 mm. Hg. This is in agreement with the finding of Hamilton (23) and of Corcoran (24).

Table 2 indicates that the left ventricular work varied from 2.89 to 6.40 kg. meters/minute, with a mean of 4.87 and a standard deviation of 0.84. Reported values for left ventricular work range from less than 0.5 kg. meters/minute in the heart-lung

TABLE 2

ANIMAL NO.	DATE	WT. LEFT VENT.	CARDIAC OUTPUT	CARDIAC OUTPUT	STROKE VOLUME	FEMORAL MEAN PRESSURE	L.V. WORK	L.V. BLOOD FLOW		L.V. O ₂ CONS.		ENERGY COST L.V. KG.	EFFICIENCY
								100 gm.	total L.V.	100 gm.	total L.V.		
		gm.	cc/min.	l/min/m ²	cc/beat	mm. Hg	kg. meter/min.	cc/min.		cc/min.		Meters/min.	per cent
I	3/21	67	2940	3.8	27	130	5.20	100	67	12.46	8.34	17.18	30.3
I	3/22	67	2160	2.8	24	125	3.67	113	76	12.92	8.66	17.81	20.6
I	3/28	67	3650	4.7	32	115	5.71	126	84	13.44	9.00	18.53	30.8
2	3/11	47	2610	4.0	16	140	4.97	138	65	19.03	8.94	18.40	27.0
2	3/14	47	2210	3.4	16	135	4.06	119	56	14.65	6.88	14.16	28.7
3	2/1	80	2840	3.6		120	4.64	146	103	18.98	15.18	31.22	14.9 ²
3	2/11	80	3840	4.8	35	118	6.16	135	95	17.54	14.03	28.90	21.3 ²
4	1/31	52	3080	4.7	26	125	5.24	132	69	18.60	9.67	19.90	26.3
4 ¹	2/2	52	6500	10.0	60	120	10.60	183	95	28.80	14.97	30.80	34.5
5	3/1	33	2520	5.1	19	125	4.28	211	70	24.04	7.94	16.34	26.2
5	3/8	33	2690	5.5		115	4.21	220	73	21.26	7.02	14.45	29.2
6	1/29	44	1770	3.1	17	120	2.89	142	63	14.35	6.32	13.01	22.2
7	3/10	32	3275	6.7	27	115	5.12	183	59	21.12	6.76	13.95	36.7
8	3/2	41	2460	4.5	21	145	4.85	192	79	19.78	8.11	16.60	29.8
8	3/17	41	2270	4.1	19	125	3.86	130	53	15.94	6.53	13.44	28.7
9	4/5	60	2770	3.4	31	128	4.82	79	54	9.58	6.61	13.61	35.4
9	4/7	60	2890	3.5	32	105	4.13	86	59	10.05	6.93	14.25	29.0
10	4/6	59	3900	5.5	30	110	5.84	155	92	19.41	11.45	23.57	24.8
10	4/8	59	4120	5.8	27	100	5.60	144	85	15.40	9.10	18.73	29.9
11	5/6	54.5	3480	4.9	32	120	5.68	110	60	13.26	7.22	14.87	38.2
11	5/9	54.5	2550	3.6		120	4.16	98	53	11.07	6.04	12.43	33.5
12	4/21	61	3320	4.6	30	135	6.10	121	74	15.28	9.32	9.17	31.8
12	4/26	61				125		124	76	15.35	9.36	19.25	
12	4/27	61	2770	3.8	25	120	4.52	112	68	13.25	8.08	16.61	27.2
13	5/23	61	3660	5.0	41	130	6.40	95	58	14.55	8.88	18.26	41.8
14 ²	5/26	54	4350	6.6		115	6.80	97	52	14.63	7.90	16.26	41.8
14	5/27	54	3120	4.7	26	110	4.67	105	57	12.57	6.78	13.96	33.4

¹ Not considered a basal animal.² Hypertrophied heart.

preparation (25) to 11.95 kg. meters/minute in the anesthetized dog (5). An average value of 5.25 kg. meters/minute has been reported in normal man (4).

The left ventricular efficiency had a mean value of 28.8 per cent with a standard deviation of 5.4. These efficiencies were lower than those reported in anesthetized

dogs (5) and slightly higher than those reported in man (4). The uniformity of the left ventricular efficiency, as contrasted to the variability of the oxygen consumption per unit weight, is further evidence that oxygen consumption per unit weight is a function of ventricular weight.

DISCUSSION

Catheterization of the coronary sinus of dogs is not a difficult procedure (2). The greatest difficulty arises from the fact that without anesthesia only trained

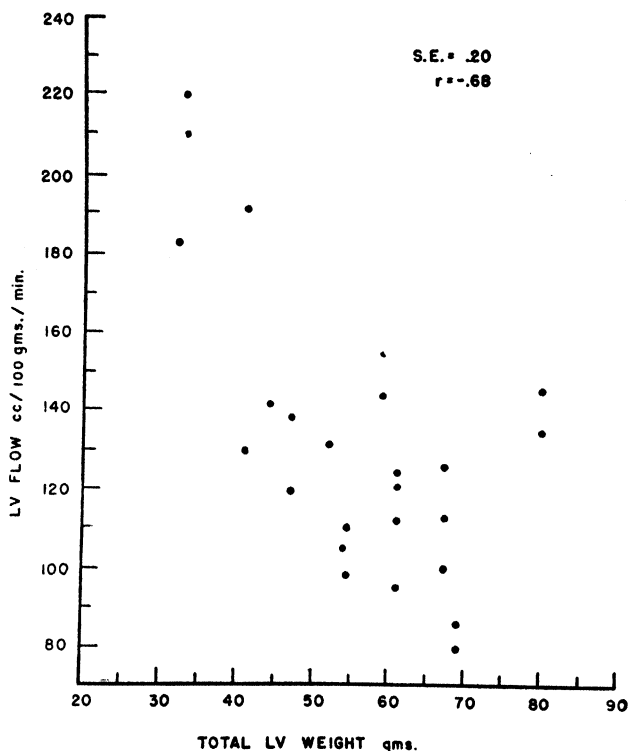


Fig. 2

animals can be used. The necessary training can usually be accomplished by 3 to 5 brief (10 to 15 minutes) training periods over a period of 4 to 7 days. The experimental procedure is not connected with any discomfort. It should be pointed out that the procedure on animals is identical with that carried out on patients under local anesthesia (4). The animals tolerate the procedure well and show no ill effects from repeated catheterizations, especially when each experiment is followed by a rest period of 1 to 2 days. Some animals have been catheterized as many as 6 times over a period of 4 to 6 weeks. In two instances where the jugular vein was thrombosed, it was necessary to insert the catheter through a vein in the foreleg.

The results in table 1 show that in the normal resting dog the oxygen content of coronary sinus blood is usually much lower than that of mixed venous blood. The left ventricular oxygen extraction ranges from 11 to 13 vol. per cent; in contrast, the oxygen extraction by peripheral tissues is only 3 to 6 vol. per cent. Similar findings have been reported in dogs and man (2, 4).

Values for left ventricular blood flow and oxygen consumption/100 gm. show marked variation (80 to 220 cc/100 gm/min. for left ventricular blood flow, 10 to 24

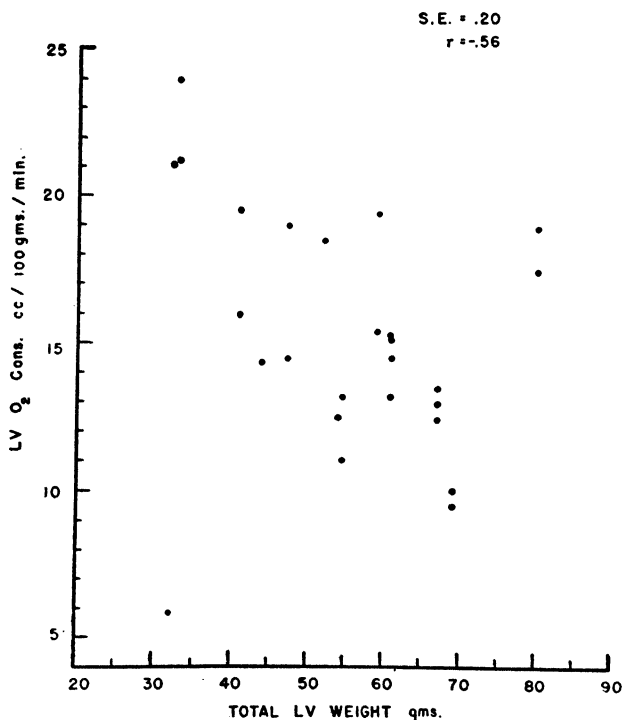


Fig. 3

cc/100 gm/min. for left ventricular oxygen consumption) (table 2). Because of the large amount of scatter present, average values are of limited significance. Eckenhoﬀ found that in anesthetized animals the values for left ventricular blood flow and oxygen consumption per unit weight were smaller and showed less variation (table 4) (3). It is probable that the use of anesthesia may partially explain the differences observed.

The blood flow and oxygen consumption of the total left ventricle have mean values of 70 cc. and 8.5 cc/minute respectively. Harrison has reported a similar average value for the oxygen consumption of the entire heart in experiments in which the coronary sinus outflow was diverted through a modified Morawitz cannula (17).

His calculations involved two assumptions which need not be made with the nitrous oxide method: *a*) that the fraction of blood drained by the coronary sinus is constant and is not altered by the insertion of the Morawitz cannula; *b*) that the coronary sinus blood is a representative blood sample for the entire heart (17). In the heart-lung preparation the oxygen consumption of the entire heart is somewhat smaller; however, the cardiac outputs observed in these preparations are much lower than those found in the normal dog (table 4) (25).

It is of interest that the standard deviations are smaller for total left ventricular blood flow and oxygen consumption than those per unit weight. This suggests that blood flow and oxygen consumption/100 gm. are related to heart weight. This relationship is graphically illustrated in figures 2 and 3, where it may be seen that an inverse relationship exists between left ventricular weight on the one hand and oxygen consumption and blood flow/100 gm. on the other. It follows, therefore, that the blood flow and oxygen consumption per unit heart weight must be greater in small ventricles. These correlations between ventricular weight and blood flow and oxygen consumption/100 gm. are statistically significant, for the correlation coefficients are approximately three times greater than the standard error (standard error of 0.20; correlation coefficients of -0.68 and -0.56). A similar relationship has been observed by Cohn in the heart-lung preparation (26). Katz, on the other hand, was unable to find such a relationship in heart-lung and isolated heart preparations (27).

The relatively higher rate of metabolism per unit of tissue in small animals is probably related to their relatively large surface area. It is generally accepted that metabolic rate is a function of the ratio of surface area to body weight (28). As ventricular weight varies directly with body weight (12), the proportion of left ventricular weight to body weight is the same in small and large animals. Consequently, the metabolism of left ventricular muscle per unit weight should be determined by the ratio of surface area to left ventricular weight. As this ratio is high in small animals, the cardiac metabolism per unit of heart weight is increased. On the other hand, in large animals the ratio of surface area to ventricular weight is low, and the cardiac oxygen consumption per unit of heart weight is decreased (table 2 and figs. 2, 3). It should be emphasized, therefore, that values for left ventricular blood flow and oxygen consumption should be considered only for the total ventricle and not per unit of weight.

The left ventricular blood flow averages 2.4 per cent of the total cardiac output (table 3). The ventricular oxygen consumption represents an average of 5.7 per cent of total body oxygen consumption (table 3). The volume of blood perfusing the left ventricular muscle is relatively small as compared to the renal or hepatic blood flow (29, 30). This indicates that the heart satisfies its oxygen requirement at rest not by a large blood flow but by a large oxygen extraction.

Some degree of correlation is present between the cardiac output and total left ventricular flow (correlation coefficient of 0.40; standard error of 0.20). It should be pointed out, however, that because of the basal state of the animal, only a limited range of cardiac output was observed. Katz has reported a good correlation between cardiac output and total coronary blood flow in a large series of experiments on hearts *in vitro* (27). Eckenhooff and associates, however, did not observe a consistent correlation in their experiments on anesthetized dogs.

The left ventricular work averages 4.87 kg. meters/minute (table 2). These values are lower than those reported by Eckenhoff in anesthetized dogs (5). It is

TABLE 3

ANIMAL NO.	DATE	CARDIAC OUTPUT	LEFT VENTRICULAR BLOOD FLOW	CARDIAC OUTPUT PERFUSING LEFT VENT. MUSCLE	OXYGEN CONSUMPTION	LEFT VENTRICULAR OXYGEN CONSUMPTION	TOTAL O ₂ UPTAKE USED BY LEFT VENT. MUSCLE
		cc/min.	cc/min.	%	cc/min.	cc/min.	%
1	3/21	2940	67	2.3	134	8.34	6.2
1	3/22	2160	76	3.5	130	8.66	6.7
1	3/28	3650	84	2.3	130	9.00	6.9
2	3/11	2610	65	2.5	145	8.94	6.2
2	3/14	2210	56	2.5	147	6.88	4.7
3	2/1	2840	103	3.6	160	15.18	9.5
3	2/11	3840	95	2.5	195	14.03	7.2
4	1/31	3080	69	2.5	160	9.67	6.0
4 ¹	2/2	6500	95	1.5	135	14.97	11.0
5	3/1	2520	70	2.8	140	7.94	5.7
5	3/8	2690	73	2.7	140	7.02	5.0
6	1/29	1770	63	3.6	105	6.32	6.0
7	3/10	3275	59	1.8	185	6.76	3.7
8	3/2	2460	79	3.2	145	8.11	5.5
8	3/17	2270	53	2.3	122	6.53	5.4
9	4/5	2770	54	2.0	175	6.61	3.8
9	4/7	2890	59	2.0	155	6.93	4.8
10	4/6	3900	92	2.4	155	11.45	7.4
10	4/8	4120	85	2.1	170	9.10	5.4
11	5/6	3480	60	1.7	160	7.22	4.5
11	5/9	2550	53	2.1	140	6.04	4.3
12	4/21	3320	74	2.2	175	9.32	5.3
12	4/26		76		155	9.36	6.0
12	4/27	2770	68	2.5	155	8.08	5.2
13	5/23	3660	58	1.6	160	8.88	5.5
14 ¹	5/26	4350	52	1.2	145	7.90	5.5
14	5/27	3120	57	1.8	155	6.78	4.4

¹ Not considered a basal animal.

probable that these discrepancies are the result of the light barbiturate anesthesia used by these workers. The values for total cardiac work as determined by Harrison

in morphinized dogs were variable but had a similar mean value (17). The marked variability in cardiac output seen in morphinized dogs has recently been emphasized

TABLE 4. COMPARISON OF VALUES OBTAINED FOR CARDIAC OXYGEN CONSUMPTION, WORK AND EFFICIENCY

METHOD	O ₂ CONSUMPTION	WORK	EFFICIENCY
	cc/100 gm./min.	kg.	
Heart-lung and similar in vitro preparations Evans (26)	Average: 6.2 Range: 4.2-7.8	(BP: 100 Hg) (Output: 200 cc/min.) Average: 0.272 (whole heart)	Usually about 5% Under maximal work conditions may ap- proach 30%
Morphinized dogs Modified Morawitz cannula Harrison (17)	Average: 8.8/100 gm. Range: 4.5-15.5	(BP: 118 mm. Hg) (Output: 890-2900 cc/ min.) Average: 3.8 Range 1.4-7.0 (whole heart)	Average: 17.2 Range: 5.7-29.4 (en- tire heart)
Anesthetized dogs N ₂ O method Eckenhoff (3) Eckenhoff (5) (normal anesthetized controls)	Average: 9.5 Average 9.8 Range: 6.8-14.4	(BP: 133 mm. Hg) (Output: 2040-5450 cc/min.) Average: 6.79 Range: 3.40-11.95 (left ventricle)	
Normal man, N ₂ O Bing (4)	Average: 7.8 Range: 7.2-8.3	Average: 5.25 Range: 4.8-6.4 (left ventricle)	Average: 22.0 Range: 19.2-24.5
Unanesthetized dogs N ₂ O method This series	Varies with weight Range: 9.58-24.04	(BP: 120 mm. Hg) (Output: 1770-4120 cc/min.) Average: 4.87 Range 2.89-6.40	Average: 28.8 Range: 20.6-38.2 (Whole left ventricle)
Examples:			
Dog 8 (11 kg.)	19.78	4.85	29.8
(L.V. 41 gm.)	15.94	3.86	28.7
Dog 9 (20 kg.)	9.58	5.84	24.8
(L.V. 69 gm.)	10.05	5.60	29.9

¹ Not calculated from total L.V. oxygen consumption.

by Seligman and associates (22). In the heart-lung preparation the work values are usually less than 0.5 kg.meters/minutes, for the cardiac outputs are usually about 200 cc/minute (25).

The left ventricular efficiency of the normal dog varies from 20 to 38 per cent

(table 2). These values agree with those of normal man (4). Lower and more variable values were found by Harrison in his experiments on morphinized dogs (17). Higher efficiencies were reported by Eckenhoff and associates for anesthetized dogs (5). The lowest efficiencies reported (average value 5%) are those on the heart *in vitro*; these are probably the results of the low cardiac outputs in these preparations (26). Numerous workers have observed a rise in cardiac efficiency when cardiac work is increased as a result of an increase in cardiac output (25). In the experiments reported in this paper an occasional high output was associated with a high ventricular efficiency (35 to 45%). There were, however, insufficient data to permit definite conclusions.

The nitrous oxide method is as yet relatively new. It was first used in anesthetized animals, where the results obtained were similar to those obtained with a bubble flowmeter (3). The data obtained in these experiments on unanesthetized animals differ in several respects from those on anesthetized animals. It is conceivable, however, that the values obtained with a flowmeter may be different from those existing in the normal animal. A flowmeter can be used only under circumstances that differ appreciably from normal. Anesthesia can cause marked changes in circulatory dynamics (22). Furthermore, to obtain blood flow per 100 gm. with a flowmeter, the amount of ventricular muscle perfused must be accurately measured and the assumption made that an equal amount of muscle is perfused in the normal animal. For these reasons it is felt that the values obtained with a flowmeter may differ appreciably from the values obtained with the nitrous oxide method in the intact unanesthetized animal.

It is believed that the data obtained in these experiments offer considerable support for the validity of the nitrous oxide technique as a method of measuring ventricular blood flow. Repeated determinations on the same basal animal on separate days gave comparable values, the average variation being 12.16 per cent of the initial flow. The left ventricular efficiencies for the entire series of animals are relatively constant and agree well with those found in normal man. Significant correlations are observed between left ventricular oxygen consumption/100 gm. and left ventricular weight, between ventricular blood flow/100 gm. and ventricular weight, and between total left ventricular blood flow and cardiac output. Such correlations over a wide range of values would not be expected in the presence of a large inconstant error in the method.

SUMMARY

The coronary blood flow, oxygen consumption and efficiency of the left ventricle have been measured in a total of 27 experiments on 14 unanesthetized dogs. The oxygen content of coronary sinus blood ranged from 2.2 to 7.3 volume per cent. The coronary arteriovenous differences averaged 11.9 volume per cent. The coronary blood flow and cardiac oxygen consumption per 100 grams of left ventricular muscle showed marked variations (from 80 to 220 cc. σ 35, and from 9.5 to 24.0 cc. σ 3.5 respectively). Since left ventricular flow and oxygen consumption per unit heart weight varied inversely with body weight, the marked variations observed were probably the result of different ratios of surface area/body weight.

The coronary blood flow and oxygen consumption of total ventricular muscle ranged from 52 to 103 cc/min. (σ 13.7) and from 6.04 to 15.1 cc/min. (σ 2.2) respectively. These values showed considerably more uniformity than those obtained per unit of heart weight. The left ventricular efficiency ranged from 20 to 38 per cent. These values were considerably lower than those previously reported on anesthetized dogs using the nitrous oxide method, but agreed well with those obtained on normal man. The high degree of correlation between left ventricular oxygen consumption and left ventricular blood flow/100 grams with left ventricular weight and between total left ventricular blood flow and cardiac output are indirect evidence of the validity of the nitrous oxide method for measurement of coronary blood flow.

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DIRECT DETERMINATION OF PARTIAL AND TOTAL TENSIONS OF RESPIRATORY GASES IN BLOOD¹

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THE interest in gas transport across the alveolar membrane has lately received new impetus from the introduction of methods by means of which it is possible to measure directly the tensions exerted by the respiratory gases in the blood. More than forty years ago A. Krogh (1) measured these tensions, and used his data to prove that alveolar gas exchange follows the simple laws of diffusion. However, his ingenious and accurate technique is too complicated for routine use, and is moreover unfit for application to man, as the apparatus must be tied into the circulation.

Three *in vitro* methods which can be applied to human problems have recently been described. Berggren (2) used the polarographic technique to determine the oxygen concentration (tension). As this technique is usable only in plasma, the red cells have to be removed first. In order to reduce oxygen consumption by the blood during centrifuging to a minimum, the latter was carried out at low temperature. However, when the blood is not fully saturated with oxygen, reducing the temperature results in a fall of oxygen tension, due to a shift to the left of the dissociation curve. In such instances the cells have to be separated at body temperature and, by adhering to a strict time schedule, the oxygen consumption can be kept low and constant (3). The method does not give information about the tensions of carbon dioxide and nitrogen. The method of Riley *et al.* (4) is based upon the same principle as that of Krogh, namely the equilibration of a small bubble of gas with a relatively large amount of blood at 37°C., followed by analysis of the bubble for oxygen and carbon dioxide. Equalization of the pressures between bubble and blood occurs with little change in the original tensions of the blood. Both equilibration and analysis are carried out in the same syringe, closed with a plunger. The total tension of the gas bubble remains atmospheric throughout the entire procedure, and the concentrations of gases in it are calculated from the change in volume after appropriate absorbents have been added. Although Comroe and Dripps apply the same principle in their method (5), it differs from that of Riley *et al.* in two important respects: *a*) equilibration between blood and bubble takes place in a glass vessel, closed with stopcocks, so that changes in the composition of the gas bubble during equilibration will not affect its volume, but might change its total pressure; *b*) after equilibration the gas bubble is removed and is introduced into a Scholander micro gas analyzer. Comroe and Dripps did not measure the total pressure of the equilibrated bubble, but rightfully assumed it to be equal to atmospheric pressure, as they were concerned only with normal arterial blood. Such assumption is, of course, not justified in the case of venous or pathological arterial blood. Furthermore they did not report upon the tensions of carbon dioxide and nitrogen.

The present study was undertaken in an attempt to extend the usefulness of Comroe and Dripps' technique. It was felt desirable to study oxygen tensions of

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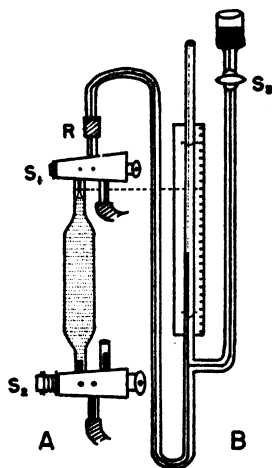
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blood over a wider range, to measure the tensions of carbon dioxide and nitrogen and to determine the total pressure exerted by the respiratory blood gases. A study was also made of the rate at which oxygen is consumed by freshly shed blood. As it was found that reproducible results could be obtained only through a meticulous technique, a detailed description of the methods used will be given.

METHODS

The tonometer (fig. 1) had a capacity of 17 cc. The plug and sidearms of the three-way stopcock *S*₁ had a bore of one mm. This stopcock was attached to the body of the tonometer in such a way that 15 mm. of the neck were formed by the capillary tubing, which ended in a smooth, olive-shaped dilatation. The presence of an appreciable length of capillary tubing below the stopcock is essential for the

Fig. 1. TONOMETER (*A*), containing blood, a small amount of mercury and a gas bubble, connected through a piece of rubber tubing (*R*) to the manometer (*B*). Diameter of the capillary of the manometer and of that of the tonometer below stopcock *S*₁ is 1 mm. Height of the tonometer, including stopcocks *S*₁ and *S*₂, is 17 cm. Height of the manometer between upper and lower bend is 26 cm. Note mercury in capillary below *S*₁, which was introduced before equilibration, and remains there during rocking of tonometer. Total tension of bubble differs from atmospheric pressure by an amount equal to difference in height of mercury meniscus in capillary below *S*₁ and that in manometer limb. Leveling bulbs not shown.



measurement of total tension, as will be shown below. The three-way stopcock *S*₂ at the foot was kept tight by means of a spring; the bore of its plug was 2 mm. A leveling bulb, filled with clean mercury, was attached to each of the two side arms of the cocks, and the tonometer, filled with mercury and 50 to 80 cu. mm. of gas of known composition was submerged in a water bath at 37°C. The following four groups of studies were made.

I. Determination of Accuracy of the Bubble Technique. In more than 50 experiments 25 cc. of blood were drawn from an arm vein into a syringe containing 0.5 cc. of a solution of 2 parts of commercial heparin solution and 8 parts of 4 per cent NaF in 0.9 per cent NaCl. The blood was mixed with a small amount of mercury and ejected into a 1100 cc. flask. The latter was thoroughly flushed with a gas mixture of known composition, closed off and rotated in a water bath at 37°C. Atmospheric pressure was maintained in the flask by opening the stopcock momentarily after 10 minutes. After 45 to 60 minutes equilibration the partial pressures of the gases in the blood were presumed to be identical with those in the gas phase.

The blood was then anaerobically transferred to the tonometer in which it displaced all but a very small amount of the mercury. Both bores of the stopcock *St* were sealed, and the initial tension of the bubble brought to atmospheric by means of the lower leveling bulb. The tonometer, with the leveling bulbs left attached, was then mechanically rocked on a frame for varying lengths of time at a rate of 24 to 32 excursions per minute. Thus the bubble was made to travel from one end of the tonometer to the other and was usually transformed into a coarse foam. As the total bubble tension after equilibration was atmospheric in these experiments, it was not measured.

The bubble was removed as described by Comroe and Dripps, with the following modifications: The tonometer was returned to the upright position with the upper stopcock still under water, and a pipette, of the type routinely used for introduction of gas samples into the Scholander apparatus (inside diameter 2.5-3.0 mm.) was attached, upside down, to the top capillary by means of clean rubber tubing. Not more than 1.5 to 2 cm. of mercury were then admitted into the pipette from the upper leveling bulb, care being taken to dislodge any air bubbles at the rubber junction. The bubble was driven into the pipette by means of the lower leveling bulb, followed by another 2 cm. of mercury. The connecting tubing was clamped off, and tubing and pipette removed. Caprylic alcohol was not used; the gas could be cleared of traces of blood that might enter the pipette by running the mercury columns and gas down to within 3 cm. of the tip, leaving the blood clinging to the wall. The best way to introduce the gas into the Scholander apparatus (model 1947) (6) was found to be the following: A rubber tip attached to the end of the pipette was firmly pressed onto the capillary of the analyzer, and the clamp at the other end released. The pipette was then gently lifted, and all air near the tip plus a small amount of mercury of the leading column were allowed to run out. The rubber tip was then again pressed onto the analyzer, and the sample admitted into the apparatus by means of the micrometer. The remainder of the analysis was as described by Scholander, except that the shaking was performed by hand, in order to keep reagents from entering the capillary. Carbon dioxide and oxygen content of 50 to 80 cu. mm. gas samples, introduced and analyzed as described, were found to be within 1.5 per cent of that of the same gas, analyzed in the conventional way in the apparatus, using 0.5 cc. samples. Immediately after collecting the first bubble a second of the same initial composition and volume was introduced into the tonometer, equilibrated and analyzed; usually a third and occasionally a fourth consecutive equilibration were carried out. Samples of 0.5 cc. of the gas in the large flask were analyzed in the Scholander apparatus. Duplicate determinations checked within 1 mm. Hg. Thus the partial tensions of oxygen and carbon dioxide in the blood as determined from analyses of the gas in the large flask could be compared with those determined from analyses of the bubble.

II. Determination of Total and Partial Tensions of Gases in Arterial Blood. In four experiments 25 cc. of blood were drawn from the femoral artery of 3 healthy young subjects who had been resting supine for 10 to 20 minutes. The syringe was lightly oiled and contained 0.5 cc. of the heparin-NaF solution. After mixing with a small amount of mercury, the blood was immediately transferred to the tonometer,

which contained a 50 to 80 cu. mm. bubble composed of 12.15 per cent O_2 and 5.25 per cent CO_2 in N_2 . The reason for the selection of this particular gas mixture will be explained under RESULTS. The tonometer was then sealed, care being taken to fill the capillary below the top cock with mercury which would remain there during the equilibration; this step is essential for the later determination of the total gas tension. After the bubble tension was brought to atmospheric as described in I, the tonometer was rocked in the water bath for 10 minutes. The total tension of the gas bubble was then measured as follows. With the tonometer in the upright position the thin thread, tied around the neck, was moved to the level of the end of the column of mercury in the capillary below S_1 . By this means the level of the upper border of the bubble was fixed. The capillary manometer B (fig. 1), fixed in a clamp, was then attached to the tonometer by means of a short piece of rubber tubing R . With stopcock S_3 open, the manometer was filled with mercury from the upper leveling bulb, so that the reservoir above S_3 was filled, and S_3 closed. Care was taken to remove all air bubbles from the rubber junction. The leveling bulb was then lowered so that the mercury in the manometer limb fell to a level below the expected total tension and S_1 closed. The bubble was then connected to the manometer by means of S_1 . This resulted in expansion of the bubble into the capillary below S_1 . Thus mercury from the manometer was prevented from falling into the tonometer, which would irreversibly decrease the volume and increase the pressure of the bubble. S_3 was then very carefully opened, the foot of the capillary mercury column brought back to the level of the thread and the total pressure of the bubble read off by determining the difference in height between the meniscus at the thread and that in the capillary manometer. Usually a second and a third reading were taken consecutively by allowing the bubble to re-expand and repeating the entire maneuver.

The following control experiments were performed with the manometer: a) The tonometer was filled with mercury and about 100 cu. mm. of room air at room temperature. The bubble was brought to atmospheric pressure as accurately as possible by means of the lower leveling bulb, and its tension repeatedly measured. The results were: -1 , -7 , $+4$, -5 , $+2$, $+5$, average -0.5 mm. Hg. In three instances too much mercury was let in, as evidenced by overshooting of the level of the thread by the mercury column. The readings were: $+29$, $+10$ and $+10$ mm. Hg. b) The capillary depressions at the menisci of tonometer and manometer are not necessarily identical: the bores are not exactly the same, and the meniscus in the tonometer is separated from the glass by a film of blood. At room temperature the pressure in the tonometer, covered with a film of blood, but otherwise empty and open to the air at S_2 , was measured. It was found to be -3 mm. Hg. A correction of 3 mm. Hg must therefore be added to all total tension measurements.

The entire manipulation with the manometer, including attaching, filling with mercury, measuring the total tension in triplicate and detaching, occupied about 10 minutes. After measurement of the total tension the bubble was equilibrated for another two minutes before being analyzed. A new bubble was then introduced, equilibrated and its total tension and composition measured.

III. Determination of Total and Partial Tensions of Gases in Mixed Venous Blood. In 5 experiments 25 cc. of blood were obtained from the right ventricle of a

dog anesthetized with sodium pentobarbital. The ventricle was reached through the anterior chest wall. The blood was collected and transferred to the tonometer as described under II. The initial composition of the gas bubble was: 5.3 to 5.5 per cent CO_2 and 6.3 to 6.8 per cent O_2 in nitrogen. The tonometer was sealed and the bubble tension brought to about minus 50 mm. Hg, as the total tension of the gases in the blood was expected to be less than atmospheric. The instrument was tilted for 10 minutes, after which both total tension and composition of the bubble were determined.

IV. Comparison of Arterial Oxygen Saturation and Tension. In a series of 11 experiments on 7 human subjects, the gas tensions of the arterial blood were determined simultaneously with its oxygen content and oxygen capacity. For the latter, the Sendroy technique was used, as modified by Roughton *et al.* (7). All determinations were made in duplicate, immediately after withdrawal of the blood. Duplicate determinations of content and capacity checked within 0.12 volume per cent. The total tension of the equilibrated gas bubble was not determined; the assumption that it was atmospheric did not influence the calculation of the partial pressure of the gases to any significant degree.

All but one of the subjects were hospital patients. Five had no lung or heart disease and were ambulatory. The sixth subject, ambulatory, had mild bronchiectasis; the seventh had severe bronchiectasis. In the latter two all but the first determinations were made after the bronchial tree had been outlined with lipiodol.

In the latter three groups of experiments a correction has to be introduced because of the dilution of blood by heparin-fluoride solution. This correction amounts to 3 mm. Hg for carbon dioxide; it is negligibly small for oxygen and nitrogen.

RESULTS

Selection of Gas Mixture for Equilibration and of Duration of Equilibration. When blood of which the $p\text{O}_2$ had been brought to 80 to 100 mm. Hg by equilibration in a large flask was equilibrated for 10 minutes with a bubble of normal alveolar air, it was found that its $p\text{O}_2$, obtained from analysis of the bubble, was within 3 mm. Hg of the expected value. Comroe and Dripps (5), using the same technique, were able to reach an accuracy of -4.0 to $+4.5$ mm. Hg. Difficulties, however, were encountered when a bubble of alveolar air was used with blood brought to a $p\text{O}_2$ of 50 to 70 mm. Hg. Equilibrium was never reached, the $p\text{O}_2$ of the bubble always being considerably higher than that of the blood. As it was hoped that prolonging the period of the equilibration would improve the results, a study was made of the influence of this factor upon gas exchange. Table 1 and figure 2 summarize the data. Although prolonging the equilibration time from 5 to 10 minutes did bring the oxygen of blood and bubble considerably closer to equilibrium, increasing the time to 15 minutes resulted only in slight further improvement. Nevertheless it was considered desirable to have access to a single gas mixture, to be used as bubble material for a fairly wide range of expected blood oxygen tensions. Although in studies on normal subjects it often would be possible to equilibrate the arterial blood with alveolar air of the subject himself, this is not convenient when dealing with patients or animals. Moreover, in patients with pulmonary pathology a considerable gradient may exist between the oxygen

tension of alveolar air and that of arterial blood. The bubble material finally selected contained 12.15 per cent O_2 , 5.25 per cent CO_2 and 82.60 per cent N_2 (pO_2 86 mm. Hg, pCO_2 37 mm. Hg, wet, $37^\circ C.$). This gas was used in all experiments on arterial blood.

Oxygen Tension. Table 2 shows that this mixture can profitably be used for blood oxygen tensions from 50 to 100 mm. Hg. Duration of equilibration in all experiments was 10 minutes. Average accuracy of the determination was $\pm 0.1 \pm 3.3$ mm. Hg. As the investigator's skill increased, more accurate results were obtained. This is illustrated in table 2 which is chronologically arranged. The degree of accuracy of the determination decreased considerably, however, when blood samples brought to a pO_2 less than 50 or more than 105 mm. Hg were analyzed with this method. The reason of this limitation, as it pertains to high blood oxygen tensions is at least in part due to the slope of the oxygen dissociation curve at high tensions as will be discussed later. At low tensions incomplete equilibration, resulting in erroneously high oxygen tensions, can be the only explanation.

TABLE 1. EFFECT OF DURATION OF EQUILIBRATION UPON GAS EXCHANGE BETWEEN BUBBLE AND BLOOD

DURATION OF EQUILIBRATION	A INITIAL BUBBLE		B BLOOD		C ¹ FINAL BUBBLE		A-C ¹ /A-B 100 (O_2)
	pO_2	pCO_2	pO_2	pCO_2	pO_2	pCO_2	
min.	mm. Hg		mm. Hg		mm. Hg		per cent
5	115.5	25.2	61.8	28.9	77.5	27.4	70.7
5	98.0	35.0	57.0	26.9	71.7	24.9	64.0
10	118.9	26.4	61.3	27.3	72.7	24.0	80.3
10	93.6	37.4	55.6	30.8	62.5	26.7	81.9
15	93.6	33.7	56.4	28.3	62.1	26.4	84.6
15	90.1	35.7	56.9	28.8	63.6	26.5	79.9

¹ Average of 3 successive bubbles.

Carbon Dioxide Tension. The results of the determination of pCO_2 were not so close to the expected tensions as was the case with pO_2 . It is believed that one of the reasons for this lies in the procedure for removing the bubble, which necessitates temporarily increasing the total bubble pressure by means of a column of Hg. This tends to send some of the highly soluble carbon dioxide into solution, and results in too low bubble pCO_2 . In later experiments this mercury column was therefore made as short as possible (15–20 mm.); in these experiments the pCO_2 was found to be within 3 mm. Hg of the expected value. The average deviation in all experiments of table 2 amounts to -5.3 ± 2.4 mm. Hg. The pCO_2 of the blood samples ranged from 25.3 to 44.0 mm. Hg. It should be pointed out that no corrections for the errors in the determination of pCO_2 and pO_2 have been introduced in the studies on arterial and venous blood that are described below.

Oxygen Consumption. The rate at which oxygen is consumed by blood at body temperature, one hour after withdrawal, was determined both at high and at low oxygen tensions (table 3). During this period the blood was equilibrated at $37^\circ C.$ in the large flask. The factor of incompleteness of equilibration was eliminated by using

as bubble a gas mixture with about the same partial tensions as those of the blood. Three bubbles were successively introduced into the tonometer, equilibrated and analyzed. At 170 mm. Hg the pO_2 fell at an average rate of 0.384 mm. Hg/minute during the first 40 minutes, that is, 1.16 mm³ O₂/100 cc. blood/minute were consumed. This rate of fall in pO_2 is of the same order of magnitude as that found by Lambertsen and Bunce (8) to occur during the first 40 minutes after sampling of normal arterial blood. Their findings, however, correspond to an oxygen consumption (in mm³) about seven times as high as was found by us. At a pO_2 of 85 mm. Hg the oxygen consumption was found to be considerably less than at high tensions. This is opposite to the results of Krogh (9) who found a fall in oxygen consumption of the blood when the animal was ventilated with high oxygen mixtures, instead of room air. In a series of normal or near-normal human arterial blood samples (not other-

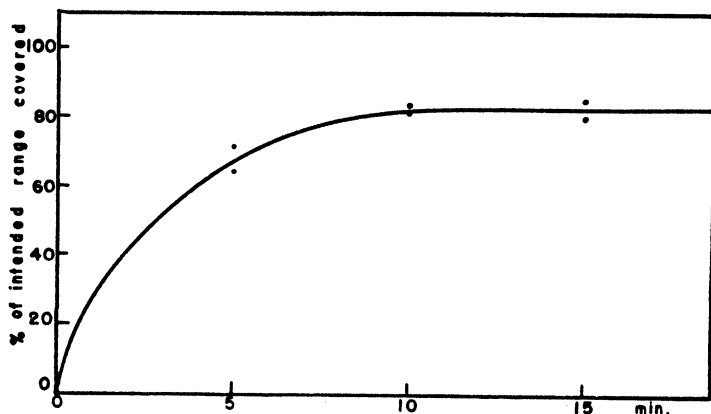


Fig. 2. INFLUENCE of duration of equilibration upon completeness of equalization of pO_2 between bubble and blood. *Abscissa*: duration of equilibration (min.); *ordinate*: percentage of intended range covered. Initial pO_2 of bubble ranged from 90.1 to 118.9 mm. Hg. Initial pO_2 of blood from 55.6 to 61.8 mm. Hg. See table 1 and text for details and discussion.

wise reported in this paper), studied immediately after sampling, the pO_2 fell at an average rate of 0.3 mm. Hg/minute in the 15 minutes between equilibration of the first and second bubble. This rate of fall is consistent with that found by Lambertsen and Bunce (8) for this period.

Total Tension and Partial Tensions of Bubble. The results of the determinations of the total tension of a 50 to 80 mm.³ bubble, in equilibrium with normal human arterial blood, are summarized in table 4. Also presented are the partial tensions of the three respiratory gases, calculated from the percentage composition of the bubble and its total tension. The total tension was several mm. Hg higher than atmospheric pressure in 3 of the 4 experiments. This does not necessarily represent the condition of the blood, *prior* to exposure to the bubble. Evidence is presented in the discussion to show that it is due to the particular equilibrium conditions prevailing in the tonometer, with respect to nitrogen.

An inevitable delay of 26 to 28 minutes occurred between the sampling of the

blood and the withdrawal of the first bubble. This interval is about 10 minutes more than occurring in experiments where the total tension was not determined, and results in a pO_2 , approximately 3 mm. Hg lower than would otherwise be found.

In table 4 similar data are also presented on right ventricular blood of two dogs, anesthetized with Nembutal. As bubble material a gas mixture was used of which the partial tensions of O_2 and CO_2 were expected to be close to those in the blood (pO_2 46 mm. Hg, pCO_2 38 mm. Hg, wet, $37^\circ C.$). With the exception of the first experiment on *dog B*, the total tension of the first bubble ranged from 22 to 27 mm. Hg below atmospheric. Here again the total tension is somewhat higher than would be expected if it were assumed that pulmonary ventilation and gas exchange were

TABLE 2. DETERMINATION OF OXYGEN AND CARBON DIOXIDE TENSIONS OF BLOOD OF KNOWN COMPOSITION

BLOOD		ERROR ¹		BLOOD		ERROR ¹	
pO_2	pCO_2	pO_2	pCO_2	pO_2	pCO_2	pO_2	pCO_2
mm. Hg		mm. Hg		mm. Hg		mm. Hg	
99.6	32.6	+3.1	-4.5	63.2	31.2	+2.6	-12.1
89.2	29.1	+0.9	-3.4	64.4	27.5	+7.1	-3.9
90.7	25.7	-4.0	-5.9	52.3	28.6	+5.9	-3.1
88.1	43.4	-2.9	-2.3	55.8	25.3	+1.2	-3.5
96.4	44.0	+2.0	-6.6	58.5	25.9	+2.8	-5.1
87.7	27.3	+1.4	-3.5	55.4	34.7	+2.4	-3.6
99.7	40.0	-3.9	-6.8	52.3	35.2	-2.0	-5.0
101.0	38.3	-3.4	-8.5	51.5	38.6	-1.7	-4.1
97.2	32.7	-3.4	-5.4	Average.....		+0.1	-5.3
98.2	40.5	-1.9	-8.6			3.3	2.4
64.2	29.8	+3.6	-5.8	Standard deviation.....			

pO_2 of initial bubble 78.2-86.3 mm. Hg in all but 5 experiments in which it was 100-108 mm. Hg.

pCO_2 of initial bubble 13.6-39.9 mm. Hg.

Duration of equilibration in the first 2 experiments 5 minutes, in all others 10 minutes.

¹ Tension of final bubble minus tension of blood. Only first equilibrated bubble is reported.

uninfluenced by the anesthesia. One explanation of this phenomenon is that the alveolar pO_2 did fall to a value considerably less than 100 mm. Hg, with a less pronounced rise in the alveolar pCO_2 . This would result in an increase in alveolar, arterial and, at equilibrium conditions, mixed venous pN_2 . For instance, if in the first animal experiment (table 4) the alveolar pN_2 were 558, instead of 733 - $(100 + 40 + 47) = 546$ mm. Hg, this would result in a rise of total tension in mixed venous blood of 12 mm. Hg. A second explanation involves the equilibrium conditions in the tonometer for nitrogen and will be discussed below.

In the fourth animal experiment of table 4 the total bubble tension was 80 mm. Hg less than atmospheric pressure. The animal was very deeply anesthetized, and was breathing slowly and at irregular intervals. The right heart blood was dark red in color; its pO_2 was only 21.3 mm. Hg. Several hours later when the respiratory rate had become normal, the pO_2 of this same dog's right heart blood had risen to 57.8 mm. Hg, coincidentally with a rise of total tension to 24 mm. Hg below atmospheric.

Oxygen Tension and Oxygen Saturation. In a series of 11 experiments on 7 human subjects, the tensions of oxygen and carbon dioxide were determined, simultaneously with the oxygen content and capacity. The Sendroy technique, as modified by Rough-ton *et al.* (7), used for the latter circumvents the errors due to drainage and conversion of some of the 'inactive' into 'active' hemoglobin, which occur when the blood is saturated in a flask. Although the small number of data precludes statistical analysis, a definite correlation in the group of normal saturations (7 experiments) did seem to exist between oxygen tension and oxygen saturation. Thus the highest oxygen tensions were encountered where the saturations were highest, and the opposite was also the case. The position of the points with respect to standard dissociation curves is shown in figure 3.

TABLE 3. OXYGEN CONSUMPTION OF SHED BLOOD

TIME AFTER WITHDRAWAL FROM FLASK	BLOOD pO_2	FINAL BUBBLE pO_2	TIME AFTER WITHDRAWAL FROM FLASK	BLOOD pO_2	FINAL BUBBLE pO_2
min.	mm. Hg	mm. Hg	min.	mm. Hg	mm. Hg
10	170.9	167.7	10	170.3	162.5
20	170.9	162.0	20	170.3	157.1
28	170.9	158.7	29	170.3	154.5
			39	170.3	155.1
10	170.7	168.7	33	83.5	85.9
21	170.7	163.8	49	83.5	87.6
31	170.7	161.8	73	83.5	80.8
11	172.3	165.0	21	84.5	86.8
31	172.3	158.2	36	84.5	86.8
			52	84.5	84.6

In the first 4 experiments the duration of equilibration was 5 minutes. pO_2 of initial bubble 171.0-172.4 mm. Hg.

In the last 2 experiments the duration of equilibration was 10 minutes. pO_2 of initial bubble 85.2 mm. Hg.

DISCUSSION

The amount of oxygen which moves from the blood into the bubble or vice versa, and the pressure exerted by the gas when equilibrium is established, can be expressed by the following equation:

$$\frac{(pO_2)_1}{P_a} V + ((pO_2)_2 - (pO_2)_3)f = \frac{(pO_2)_3}{P_b} V, \text{ in which}$$

$(pO_2)_1$ = initial oxygen tension of bubble (mm. Hg)

$(pO_2)_2$ = initial oxygen tension of blood (mm. Hg)

$(pO_2)_3$ = oxygen tension of bubble and blood after equilibration (mm. Hg)

P_a = initial total tension of bubble (mm. Hg)

P_b = total tension of bubble after equilibration (mm. Hg)

V = volume of bubble (mm.³)

f = change in oxygen content (mm.³) of the blood in the tonometer per one mm.

Hg change in oxygen tension. This factor, which depends upon the slope of the oxygen dissociation curve, has the following values for 17 cc. of blood: in the 20 mm. Hg range, 85.0; in the 60 mm. Hg range, 13.6; in the 100 mm. Hg range 3.4; above 100 mm. Hg, 0.51.

TABLE 4. TOTAL TENSION AND PARTIAL TENSIONS OF GASES IN NORMAL ARTERIAL (HUMAN) AND RIGHT VENTRICULAR (DOG) BLOOD

SUBJECT (ARTERIAL)	TIME OF REMOVAL OF BUBBLE AFTER SAMPLING	ATMOSPHERIC PRESSURE	TOTAL TENSION ¹	PARTIAL TENSION		
				pO ₂	pCO ₂	pN ₂
	min.	mm. Hg	mm. Hg		mm. Hg	
R. 1st bubble	28	744	+9, +5, +10 (av. +8)	98.7	38.1	568.2
2nd bubble	58		0	82.5	27.0	587.5
3rd bubble	88		-8, -2, +2 (av. -1.5)	67.5	33.9	594.1
R. 1st bubble	28	746	+13	93.9	39.5	578.6
2nd bubble	60		-10, -15 (av. -12.5)	88.1	35.7	575.2
M. 1st bubble	29	744	+3, -3, -2 (av. -1)	96.9	38.0	561.1
2nd bubble			-1, 0 (av. -0.5)	94.0	35.5	567.0
R _i . 1st bubble	26	741	+16, +17 (av. +16.5)	99.0	38.7	556.3
2nd bubble	55		+3, +12, +7 (av. +7)	91.0	41.4	568.6
DOG (RT. VENTR.)						
A. 1st bubble	26	733	-20, -21.5, -23 (av. -22)	58.8	46.8	558.4
2nd bubble	59		-13, -16 (av. -15)	49.4	52.4	569.2
A. 1st bubble	30	732	-38, -21, -21 (av. -27)	56.0	46.4	555.6
A. 1st bubble	29	744	-23	52.5	47.1	574.4
B. 1st bubble		743	-80, -81 (av. -81)	21.3	46.2	544.5
B. 1st bubble	20	743	-24, -24 (av. -24)	57.8	43.9	566.3
2nd bubble	45		-23	54.8	50.4	564.8

¹ With reference to prevailing atmospheric pressure.

Similar equations express the exchange of carbon dioxide and of nitrogen between bubble and blood. The factor f for CO₂ is about 68, that for nitrogen 0.34.

In order for any technique to be useful and dependable the blood pO₂ should not change more than one mm. Hg as a result of the equilibration. It can be calculated from the above equation that, using a bubble of 70 cu. mm., the initial oxygen tension of the bubble, (pO₂)₁, should therefore be within 39 mm. Hg of that of the

blood, $(pO_2)_2$, when the latter is in the 100 mm. Hg range. The difference between $(pO_2)_1$ and $(pO_2)_2$ can even be greater at lower blood oxygen tensions. A bubble with an initial oxygen tension of 85 mm. Hg as used in the experiments described here can therefore safely be used for oxygen tensions of 100 mm. Hg or less. However when the tension is much higher than 100 mm. Hg, $(pO_2)_1$ should be within 6 mm. Hg of $(pO_2)_2$ to avoid an experimental error of more than one mm. Hg. It follows that the technique described is not suitable for the accurate determination of these high tensions at which the slope of the dissociation curve is flat, and only the amount of dissolved oxygen varies.

In the case of carbon dioxide the great solubility of this gas in blood allows considerable differences to exist between bubble and blood tension with hardly any change in the latter during equilibration.

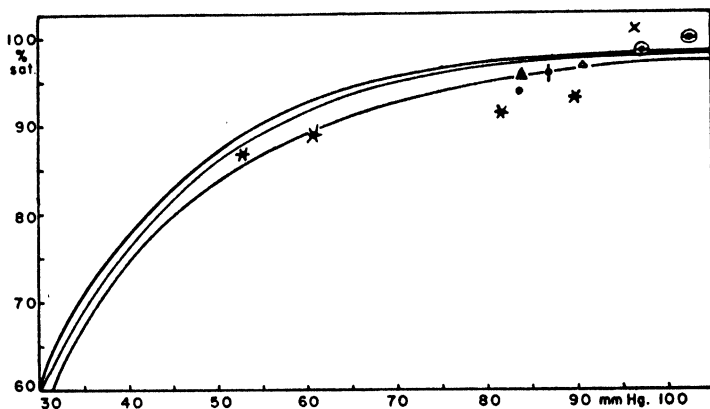


Fig. 3. OXYGEN TENSIONS AND SATURATIONS of arterial blood of 7 subjects (pCO_2 36.1-42.5 mm. Hg). The position of the points is compared with three accurately determined dissociation curves ($pCO_2 = 40$ mm. Hg) obtained from the literature. (Upper two curves: from *J. Biol. Chem.* 59: 353, 1924. Lower curve: from *Handbook of Respiratory Data in Aviation*, Washington D. C., 1944.) For discussion, see text.

The situation with respect to nitrogen is similar to that described for oxygen at high tensions. The critical value for the difference between bubble and blood nitrogen tension, $(pN_2)_1 - (pN_2)_2$, is only 4 mm. Hg. Therefore, to take a specific example from our studies, when a bubble of 70 cu. mm. volume with a nitrogen tension of 578 mm. Hg (82.6 per cent N_2 in dry gas) is equilibrated with normal arterial blood with a nitrogen tension of about 563 mm. Hg, the final nitrogen tension of blood and bubble will be 6 mm. Hg higher than the original blood tension. This will not significantly affect the percentages of O_2 and CO_2 in the bubble, but it will give the false impression that the total tension of the blood gases is higher than atmospheric pressure. In our studies the average total tension of the blood gases of normal arterial blood was found to be plus 5.5 mm. Hg (table 4). Similar conditions prevail in the experiments on mixed venous blood (table 4). Although it is difficult to determine exactly the magnitude of the 'nitrogen effect' in the case of venous blood, it probably results in a final total tension which is 3 to 6 mm. Hg too high.

Thus far it has been assumed that 10 minutes are sufficient to abolish any pressure gradient between bubble and blood. This may not be strictly true for nitrogen which has a much smaller 'invasion coefficient' than oxygen and carbon dioxide (Bohr). In the experiments described, in which the initial bubble pN_2 was always higher than the initial blood pN_2 , this again would tend to give a somewhat higher total bubble tension than the sum of the tensions of the respiratory gases in the blood, although it would not appreciably affect the oxygen and carbon dioxide percentage of the bubble.

Incompleteness of nitrogen equilibration under certain conditions has been found by Riley *et al.* (4). In their technique the blood is equilibrated with a small bubble, which remains at atmospheric pressure. Changes in the distribution of gases between bubble and blood manifest themselves therefore in changes in bubble volume. Whereas in the Riley method a small remaining nitrogen gradient will not disturb the determinations of oxygen and carbon dioxide tensions any more than it will in the method described in this paper, the situation is different when the total tension of the blood gases is significantly different from atmospheric pressure. When venous blood, or arterial blood obtained at high altitude, is equilibrated with a gas bubble, the total tension of which is kept at atmospheric pressure at sea level, no equilibrium of any of the gases can ever be obtained, and the bubble will continue to shrink, as the gases are forced into the blood. For instance, if arterial blood obtained at 4000 feet (bar. press. 656 mm. Hg) and 90 per cent saturated with oxygen, is equilibrated at sea level, by means of the Riley technique, with a bubble of which the pO_2 is 60 mm. Hg and the pCO_2 40 mm. Hg, the oxygen and carbon dioxide tensions are about equalized to begin with. However there is a nitrogen gradient of +100 mm. Hg from bubble to blood. Nitrogen will be forced into the blood and the bubble will shrink. This results in a rise of tension of the other two gases which will therefore be forced into solution. The bubble analysis for these gases would be accurate only if the nitrogen did not move at all. The faster nitrogen is forced into the blood, and the longer the blood is exposed to the bubble, the more will the partial pressures of CO_2 and O_2 in the bubble differ from those in the original blood. The only solution to this difficulty is to perform the equilibration at the total tension which obtained when the blood was collected. In the method described in this paper, a total blood gas tension higher or lower than atmospheric pressure does not interfere with the establishment of true equilibrium. The volume of the bubble remains constant; its total tension adjusts itself accordingly to the partial tensions prevailing in the blood.

Oxygen Saturation and Oxygen Tensions. Comroe and Dripps (5) found in their study on 13 normal resting subjects, that the arterial oxygen tension ranged from 93.0 to 100.6 mm. Hg. Recently Wood (10) has reported the arterial oxygen saturation of 29 normal resting individuals. The oxygen capacity was determined with the Sendroy-Roughton technique. The saturation ranged from 94.1 to 101.0 per cent. If each of the individuals studied by Wood had an arterial oxygen tension in the range found by Comroe and Dripps, this would mean that the course of the oxygen dissociation curve at high oxygen tensions is quite different in various individuals: a range of only 7 mm. Hg is accompanied by one of as much as 7 per cent saturation. On the other hand, it is possible that the arterial oxygen tension varies more in healthy

persons than is apparent from Comroe and Dripps' figures. The data reported here lend support to the latter possibility. Although the number of experiments is rather small, a definite correlation does seem to exist between arterial oxygen saturation and oxygen tension in the studies, where the saturation was within normal limits. Thus the highest oxygen tensions were encountered where the saturation was highest, and the opposite was also the case. The normal oxygen tensions varied from 83.6 to 102.6 mm. Hg, a range not unlike that reported by Lilienthal *et al.* (11); the normal saturations from 93.6 to 100.6 per cent.

It is not certain whether the variability in normal arterial oxygen tension is due to a parallel variability in alveolar oxygen tension, or to individual variations in the amount of blood shunted past poorly ventilated alveoli or added to the pulmonary veins from the bronchial system.

SUMMARY

The partial and total tensions exerted by the respiratory gases in blood were determined by means of a direct method. The principle of the method consisted of the equilibration of a 50 to 80 mm.³ bubble of gas of known composition with 17 cc. of the blood in a tonometer of the type described by Comroe and Dripps (5). The tonometer was rocked at 37°C. for varying lengths of time in order to produce equalization of the partial tensions of bubble and blood. A capillary manometer was then attached to the tonometer, and the total tension of the bubble at constant volume was measured. After an additional two minutes of equilibration, the bubble was collected in a pipette between mercury and analyzed in a Scholander micro gas analyzer.

In more than 50 experiments the accuracy of the bubble technique was determined by analysis of the bubble equilibrated with blood of known oxygen and carbon dioxide tensions. Equilibration of 50 to 80 mm.³ of gas originally containing 12.15 per cent O₂ and 5.25 per cent CO₂ in N₂ for 10 minutes permitted the determination of pO_2 with an error of $+0.1 \pm 3.3$ mm. Hg and of pCO_2 with an error of -5.3 ± 2.4 mm. Hg, when the pO_2 of the blood ranged between 50 and 105 mm. Hg. The rate at which oxygen is consumed by blood at body temperature, one hour after withdrawal, was determined at pO_2 of 170 and 85 mm. Hg. At high pO_2 this rate amounts to 0.384 mm. Hg per minute, or 1.16 mm.³ O₂ per 100 cc. blood per minute. At low pO_2 the rate is very much less. In 4 experiments the total and partial tensions of the gases in arterial blood of 3 normal subjects were measured. The average total tension was 5.5 mm. Hg higher than the prevailing atmospheric pressure. The pO_2 ranged from 93.9 to 99.0 mm. Hg, the pCO_2 from 38.0 to 39.5 mm. Hg. In 5 experiments the tensions of right ventricular blood of 2 anesthetized dogs were measured. In 4 of these studies the total tension was 22 to 27 mm. Hg less than atmospheric pressure. The pO_2 ranged from 52.5 to 58.8 mm. Hg, the pCO_2 from 43.9 to 47.1 mm. Hg. In the fifth study the total tension was -80 mm. Hg, with a pO_2 of 21.3 mm. Hg, a pCO_2 of 46.2 mm. Hg. In 11 experiments on 7 human subjects, the gas tensions of the arterial blood were determined simultaneously with the oxygen saturation. The oxygen capacity for the latter was measured with the Sendroy-Roughton technique. A definite correlation was found between oxygen tension and oxygen satura-

tion in the group of seven normal saturations. Thus the highest tensions were accompanied by the highest saturations, and the opposite was also the case.

The equilibrium conditions in the tonometer are critically discussed, and their bearing upon the accuracy of the technique is pointed out.

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EFFECT OF AURICULAR FIBRILLATION ON THE CORONARY BLOOD FLOW¹

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THE only information available on the effect of auricular fibrillation on the coronary circulation is the work of Miller, Smith and Graber (1) who, measuring the coronary sinus outflow with a Morawitz-Zahn cannula in dogs, concluded that in auricular fibrillation the rate of the coronary circulation either remains fairly constant or is moderately accelerated. Because of the paucity of information, this problem was thought to be worthy of reinvestigation with more precise means of measuring blood flow.

METHOD

Sixteen dogs weighing from 12 to 33 kg. were used. They were given subcutaneously 2 cc. of a 2 per cent morphine sulfate solution; then they received intravenously 1.25 cc. to 1.5 cc. of a 20 per cent sodium barbital solution per kilogram of body weight. Under artificial respiration, the chest was opened through a midsternal incision and the heart suspended in a pericardial cradle. A segment of the left anterior descending coronary artery was dissected. The blood was rendered incoagulable by the intravenous administration of an initial dose of 5 mg. heparin/kilogram, then 3 mg./kilogram every half hour. The mean coronary blood flow was measured and recorded with an electromagnetic rotameter (2, 3). A cannula was inserted in the left common carotid artery; in some experiments, it was introduced down to the root of the aorta in the neighborhood of the coronary ostia. As shown in figure 1, when the screw clamps placed in *E* and *G* were open and the screw clamp placed in *F* was closed, the blood was allowed to flow via the carotid cannula *A*, through the flowmeter *B*, into the cannulated coronary artery *C*. When the screw clamps *E* and *G* were closed and the clamp *F* was open, the blood flowed from the carotid cannula *A* via the short circuit into the cannulated coronary artery *C*. In this manner, a zero flow was recorded without producing myocardial ischemia. An optical manometer of the Gregg type, *D*, was used to record the mean arterial blood pressure. The flowmeter had been previously calibrated, according to the technique of Shipley and Crittenden (2, 3) with liquids of viscosities covering the range of viscosities of the dog blood in such acute experiments. Auricular fibrillation was induced by

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electrical (60 cycles/second alternating current) stimulation of minimal intensity applied to the right or left auricular appendage through two fishhook electrodes 0.5 to 1 cm. apart. As the ventricular rate could not be obtained from the tracing of either the mean arterial blood pressure or the coronary flow, an electrocardiogram (*Lead II*) was recorded in 17 out of the 69 bouts of auricular fibrillation.

RESULTS

Sixty-nine bouts of auricular fibrillation induced in 16 dogs were studied. Electrical stimulation was applied from 5 to 300 seconds. In some cases, fibrillation stopped as soon as stimulation was discontinued; in other cases it persisted for as long as approximately 9.5 minutes after stimulation was discontinued. No difference

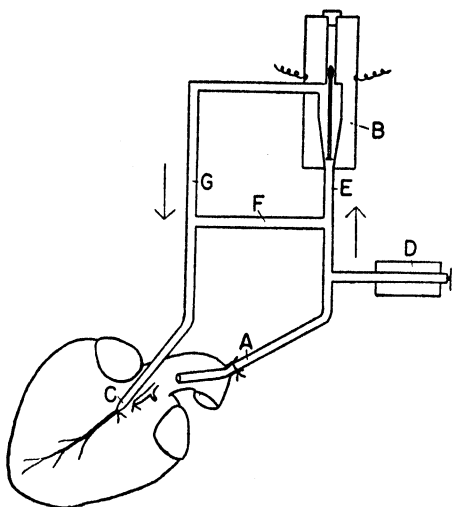


Fig. 1. SCHEMATIC DRAWING OF APPARATUS used to measure and record mean arterial blood pressure, and mean coronary blood flow in left anterior descending coronary artery.

in the pattern of the cardiovascular reactions was found between the bouts of fibrillation maintained by continuous stimulation and those that persisted after stimulation discontinued. Three illustrative examples of bouts of auricular fibrillation, all induced in the same dog, are shown in figures 2 and 3.

Figure 2*A* shows a very short bout of auricular fibrillation which lasted 12 to 13 seconds and stopped as soon as electrical stimulation of the auricular appendage was discontinued. Before the induction of fibrillation, the mean arterial blood pressure oscillated around 130 mm. Hg and the mean coronary flow was about 41 cc/minute. As soon as the electrical stimulation was applied, auricular fibrillation ensued (fig. 2*A*, first arrow).

Blood pressure and coronary flow fell very markedly and both remained below control levels throughout the bout of fibrillation. As soon as stimulation was discontinued, fibrillation stopped (fig. 2*A*, second arrow) and both blood pressure and coronary flow increased. The blood pressure rose to a maximum of 140 mm. Hg, then decreased to a minimum of 110 mm. Hg and then returned and remained around 130 mm. Hg. The coronary blood flow rose to a maximum of 76 cc/minute, then decreased to a minimum of 35 cc/minute and then rose again and remained at a value of about 40 cc/minute. Figure 2*B* shows a bout of auricular fibrillation which lasted 55 seconds and stopped as soon as electrical stimulation was discontinued. During the control period, the blood pressure ranged around 125 mm. Hg and the mean coronary flow around 39 cc/minute. At the onset of fibrillation (fig. 2*B*, first arrow), both blood pressure and coronary flow fell markedly. The blood pressure then returned toward con-

trol level without reaching it, but the coronary flow ranged around its control level. At the termination of stimulation (fig. 2*B*, second arrow), auricular fibrillation ceased immediately and both blood pressure and coronary flow rose above control values, the blood pressure rising to a maximum of 140 mm. Hg and the coronary flow rising to a maximum of 63 cc/minute. Then they both decreased to values slightly below control values, a minimum of 110 mm. Hg and 34 cc/minute for blood pressure and blood flow, respectively. Finally they rose to stable levels of around 130 mm. Hg and 39 cc/minute. Figure 3 is the continuous record of a bout of auricular fibrillation that lasted 10 minutes and 19 seconds. Fibrillation was induced at the first arrow by electrical stimulation. At the second arrow, 36 seconds later, the stimulation was discontinued, but fibrillation persisted for another 9

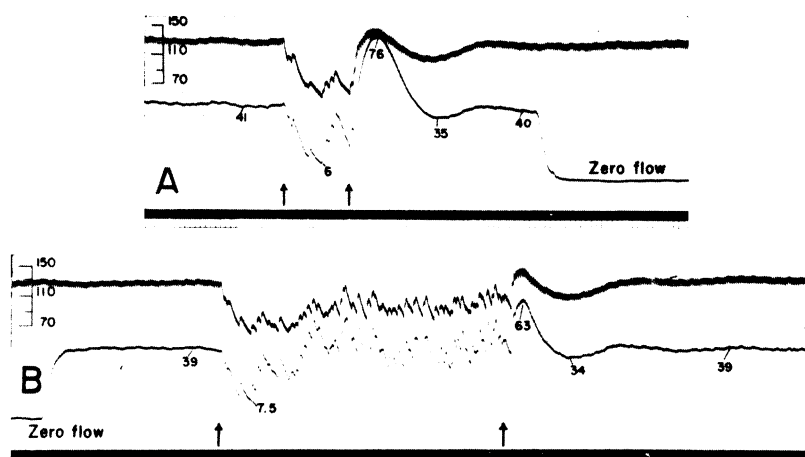


Fig. 2. *A* is bout of auricular fibrillation lasting about 12 seconds. *B* is bout of auricular fibrillation lasting 55 seconds. *Upper tracing*: mean aortic blood pressure, scale in mm. Hg. *Lower tracing*: mean blood flow in the left anterior descending coronary artery; numerals indicate blood flow in cc/minute (time in seconds). At first arrow is beginning of electrical stimulation and induction of auricular fibrillation. At second arrow is end of stimulation and auricular fibrillation.

minutes and 43 seconds. It stopped spontaneously, as stated, 10 minutes and 19 seconds after its onset (fig. 3, third arrow). During the control period, the mean arterial blood pressure was around 140 mm. Hg and the coronary flow around 41 cc/minute. At the onset of fibrillation, both blood pressure and coronary flow fell abruptly and markedly, then both returned toward control levels. Occasionally the blood pressure and more often the coronary flow rose above control levels. When fibrillation stopped, both coronary flow and blood pressure rose markedly above control values, then they decreased to a minimum of 120 mm. Hg and 43 cc/minute, respectively. Within a few seconds, the blood pressure rose to 145 mm. Hg and the coronary flow to 48 cc/minute, values above control levels. The blood pressure then reverted to slightly below control level, whereas the coronary flow remained above its control value, approaching it gradually. However, about 6 minutes after the cessation of fibrillation, the coronary flow was still 45 cc/minute, a value above its

control level, although the blood pressure was 130 mm. Hg, a value slightly below control level.

Essentially similar reactions in mean arterial blood pressure and coronary flow were observed in all experiments. At the onset of auricular fibrillation, blood pressure and coronary flow decrease markedly and suddenly. Then they both rise toward or to control levels. If fibrillation persists long enough, they may even rise above control levels, at least temporarily. It must be pointed out that, when rapid fluctuations occur in the coronary flow, there must be a definite overshooting in the tracing of the coronary flow because of the inertia of the rotameter. Therefore it is not possible to quantitate exactly the blood flow at such peaks in the tracing of the coronary flow

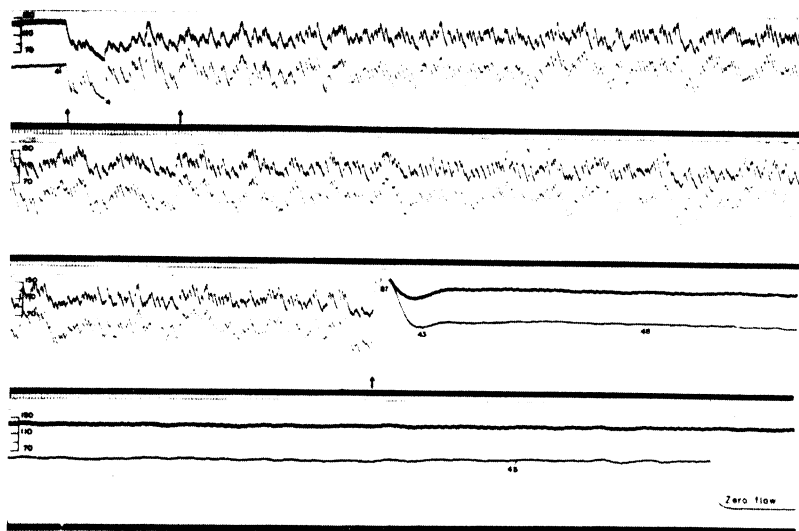


Fig. 3. CONTINUOUS RECORD OF BOUT of auricular fibrillation lasting 10 minutes and 19 seconds. *Upper tracing:* mean arterial blood pressure, scale in mm. Hg. *Lower tracing:* mean blood flow in the left anterior descending coronary artery; numerals indicate blood flow in cc/minute (time in seconds). At first arrow is beginning of stimulation and onset of fibrillation. At second arrow is cessation of fibrillation. At third arrow is spontaneous cessation of auricular fibrillation.

As soon as fibrillation stops, both blood pressure and coronary flow increase and rise above control levels, especially the coronary flow. Then they decrease, in many cases even below control levels. Finally, both blood pressure and coronary flow come back to their control levels. The coronary flow may even rise above its control level again, and, when the bout of fibrillation is prolonged, it returns slowly to its control level.

In the 17 experiments in which an electrocardiogram was recorded, the control ventricular rates ranged between 110 and 200/minute; during auricular fibrillation, the increase in ventricular rate amounted to between 50 and 100 per cent of the original rate. The cardiovascular reactions observed were independent of the initial ventricular rate and there was no relation noted between the ventricular rate and the pattern of change in coronary flow and blood pressure during auricular fibrillation.

DISCUSSION

Certain definitive conclusions can be drawn from the reported studies. Thus, when during the bout of auricular fibrillation, the coronary flow is at or even above control level and the blood pressure is below control level, there must be a decrease in the resistance of the coronary bed. The same is true when, after auricular fibrillation has subsided, the coronary flow remains above its control value when the blood pressure has returned to its control level. Whether or not the decrease in the resistance of the coronary bed is caused by a decrease in the extra-vascular support or by active dilatation of the coronary bed, has not been ascertained.

Although the rest of the cardiovascular reactions observed are susceptible of a logical explanation, an attempt to interpret them without concomitant measurement of cardiac output must of necessity remain speculative. It seems probable that, at the onset of fibrillation, the cardiac output decreases and as a result the arterial blood pressure falls. The decrease in coronary flow may very well be explained by this drop in aortic pressure. Within a few seconds, compensatory mechanisms seem to set in. These mechanisms probably include cardiac adjustments as well as peripheral adjustments leading to an increase in cardiac output toward its control level or above and a rise in the mean arterial blood pressure toward and occasionally above its control level, in the longer bouts of fibrillation. As fibrillation stops, it seems probable that the cardiac output increases which, with the peripheral vasoconstriction that occurred during fibrillation, leads to a rise in arterial blood pressure. Both factors contribute to the sudden and sharp increase of the coronary blood flow. Then the blood pressure returns to normal whereas, at least in the longer bouts of fibrillation, the coronary flow remains elevated above its control level. As mentioned previously, it is impossible to state whether or not this increase in coronary flow is caused by a decrease in the extravascular support or by an active vasodilatation of the coronary bed. The latter however seems much more probable. However, it cannot be stated whether or not this active vasodilatation is caused by the effect of the metabolites that accumulated in the myocardium during fibrillation or by an increase in the cardiac output and cardiac work resulting from the bout of fibrillation (4). Of course, both factors, accumulation of metabolites in the myocardium and increase in cardiac work, may be operating. At any rate, it seems that a bout of auricular fibrillation during which the ventricular rate is high calls for an increase in the coronary flow. In a heart with coronary sclerosis, this increase may be neither possible nor sufficient to meet the demands.

SUMMARY

Sixty-nine bouts of auricular fibrillation induced in 16 dogs were studied as to their effect on the mean arterial blood pressure and coronary flow. At the onset of auricular fibrillation, there is a marked drop in blood pressure and coronary flow. Then, within a few seconds, both blood pressure and coronary flow return toward their control levels, but whereas the coronary flow may, at least temporarily, return to or even rise above its control level, the blood pressure very seldom reaches it. As soon as fibrillation stops, both coronary flow and blood pressure rise above control

levels, then come down progressively to their control level, but the blood pressure may come back to its control level before the coronary flow. The mechanisms and significance of the cardiovascular reactions observed have been discussed.

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COMPARISON BETWEEN THE CARDIAC INPUT MEASURED WITH A ROTAMETER AND OUTPUT DETERMINED BY THE DIRECT FICK METHOD IN OPEN-CHEST DOGS

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THE rotameter¹ is obtainable in a size capable of measuring the total venous return to the heart in moderate-sized dogs. However, few papers have been published in which the rotameter has been used to measure cardiac input (1) and none in which simultaneous determinations of cardiac input and output have been made. This paper presents data comparing the cardiac input measured with a rotameter and the output determined by the direct Fick method. In addition, data are included on factors affecting the calibration of the instrument.

METHODS

The techniques used in this series of experiments are the same as those reported in a previous paper (2).

RESULTS

Table 1 presents data on the measured cardiac input with the rotameter and the output determined by the direct Fick method. The coefficient of correlation is $r = +0.96$. The average difference is ± 8.9 per cent with a range from -29.3 to $+25.8$ per cent.

In making the calibrations a series of cannulae were selected that would give rates of flow over the range occurring experimentally. To establish a given point, 2 and often 3 determinations were made for each flow rate.

During any particular calibration, the points at any flow rate were reproducible with but negligible error for either blood or saline. On successive days with saline or on the same day with different specimens of blood the curves deviated considerably. A saline curve might differ by as much as 15 per cent from one taken the previous day. However, this represents the extreme limits found (fig. 1). More commonly the difference was about 5 per cent. In the case of blood the deviation was greater (fig. 1) and marked differences occurred in the shape of the curve in contrast to saline where the shape of the curve was relatively constant. Greater variations in the blood calibration curves is due to differences in viscosity caused by temperature (fig. 2) and

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¹ The rotameter used in these experiments was the electronic recording type and was obtained from Mr. Clifford Wilson of Eli Lilly and Company.

the variable composition of blood (fig. 3). Although with some bloods, a considerable temperature change had a negligible effect on the calibration curves.

TABLE I. COMPARISON OF DIRECT FICK (100%) AND ROTAMETER METHODS OF MEASURING CARDIAC OUTPUT AND INPUT

DOG NO.	DIRECT FICK	ROTAMETER	PERCENT-AGE DIFFERENCE	DOG NO.	DIRECT FICK	ROTAMETER	PERCENT-AGE DIFFERENCE
	cc/min.	cc/min.			cc/min.	cc/min.	
CO-6	154	160	+3.8	CO-11	578	515	-10.8
	189	230	+21.6		668	540	-19.1
	280	330	+17.8		689	600	-12.9
	214	195	-8.8		310	390	+25.8
	142	140	-1.4	R-1	180	195	+8.3
CO-7	485	435	-10.3		396	385	-2.7
	371	390	+5.1		283	200	-29.3
	695	510	-26.6		262	290	+10.6
CO-9	583	500	-14.2	R-3	201	200	-4.9
	562	500	-11.0		177	170	-3.9
	472	450	-4.6		217	209	-3.6
	525	500	-4.7		500	495	-1.0
	635	620	-2.3	R-4	295	280	+1.6
CO-10	737	710	-3.6		840	750	+10.7
	375	370	-1.3		735	710	+3.4
	396	360	-9.0		615	600	+2.4
	386	340	-11.9		427	410	+3.9
	312	290	-7.0				
	885	900	+1.6				

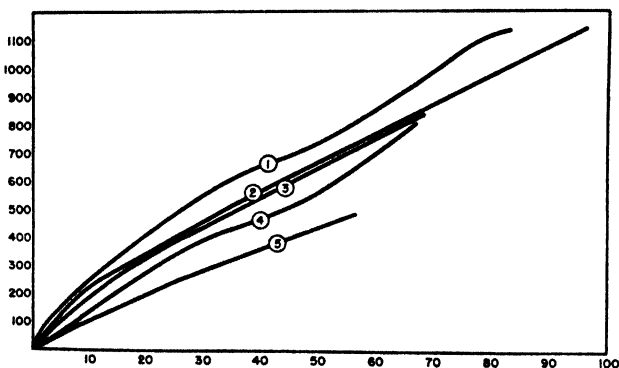


FIG. 1. THE ABSCISSA GIVES METER READINGS AND THE ORDINATE CC/MIN. Curves 1 and 2 represent the extreme limits found for saline and curves 3, 4 and 5 represent extremes for the blood curves.

DISCUSSION

According to calibration curves shown by Crittenden and Shipley (3) for instruments of this type, viscosity difference ranging from water to an acacia solution

(specific viscosity of 4.5) have so slight an effect on the calibration that viscosity changes occurring during an experiment may be disregarded. On the other hand, in the original paper on the rotameter, Gregg *et al.* (4) demonstrated viscosity changes

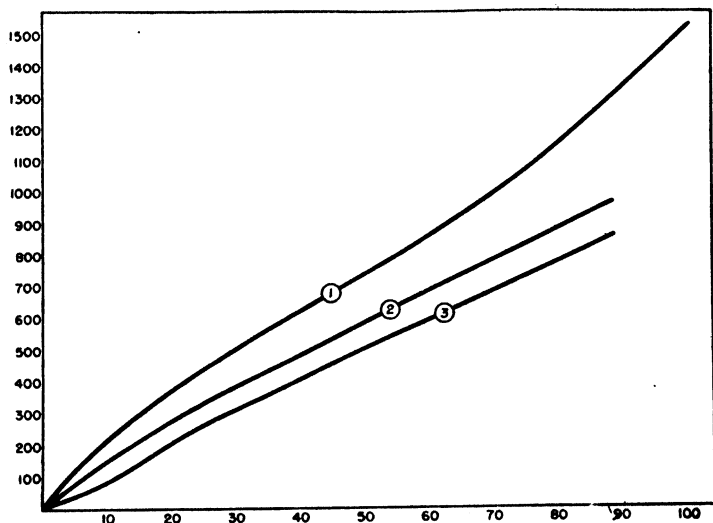


FIG. 2. THE ABSCISSA GIVES METER READINGS AND THE ORDINATE CC/MIN. Curve 1 represents the lack of effect of temperature changes in that the curve was the same for blood at 38°C. and 22°C. Curve 2 is a calibration with blood at 40°C. Curve 3 is the same blood as curve 2, but at 18°C.

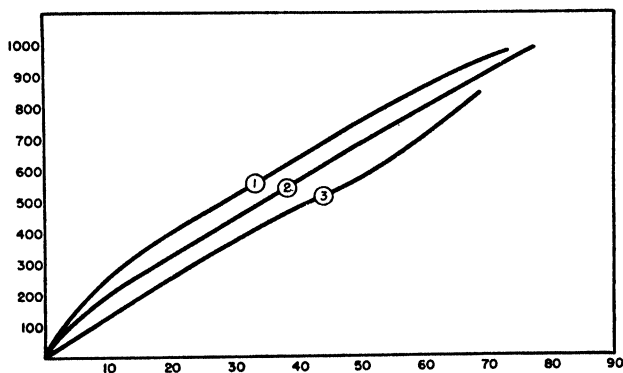


FIG. 3. THE ABSCISSA GIVES METER READINGS AND THE ORDINATE CC/MIN. Curve 1 is saline, curve 2, 50 per cent saline, 50 per cent blood, curve 3, whole blood (same blood as used in curve 2).

to be of great importance regardless of whether or not the changes resulted from differences in temperature, hemodilution or concentrations.

The calibration curves obtained in this study are different in shape from those published (3, 4). The 2 chief differences are: 1) The curves previously published are all concave to the abscissa while those reported here are convex at low rates of flow.

2) The previously published curves are smooth and regular in shape while here they are variable. The type of curve shown in figure 1, curve 4, was not unusual, occurring in varying degrees with blood from several dogs.

These changes in the form of the curves with different bloods do not prevent the use of the rotameter with high reliability so long as an adequate number of points for each experiment are determined to establish the true curve. If this is not done an appreciable degree of error will be introduced at certain flow rates.

The suggestion of Gregg *et al.* (4) that it is possible to calibrate the rotameter by taking one flow and float reading and observing the position of this one point on a previously made series of *in vitro* calibration curves from blood of different viscosities should not be used in view of the data presented.

SUMMARY

The cardiac input measured with a rotameter agrees closely with the output determined by the direct Fick method. In calibrating the rotameter it is necessary that an adequate number of points be determined with the temperature of the blood approximately the body temperature of the animal used because of the variability between flow and the meter reading at different flow rates with some bloods.

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INHIBITION OF RAT BRAIN CHOLINESTERASE BY β -CHLORINATED AMINES¹

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THE role which acetylcholine plays in the nervous system is not yet satisfactorily elucidated. In a search for the mechanism of convulsions, however, acetylcholine and the enzyme systems concerned with its metabolism cannot be ignored. The β -chlorinated amines are potent convulsants and the structural requirements necessary for convulsant activity have been determined (1). The inhibitory effect of a few β -chlorinated amines on cholinesterase has been reported (2, 3) but it was of interest to study systematically a series of these compounds in an attempt to determine the structure necessary for inhibitory activity and to see if there was any correlation with the convulsant structure found previously (1). The data leading to the discovery of such a correlation form the basis of a part of this report. Also reported are results of studies on the effects of brain cholinesterase of various other convulsant and anticonvulsant drugs.

METHODS AND RESULTS

Cholinesterase was followed by the manometric method of Ammon (4) as described by Nachmansohn and Rothenberg (5). The brains of 150 to 250-gm. Sprague-Dawley rats were used as the source of enzyme. The animals were killed by decapitation, the brains quickly removed and the medulla and cerebellum cut away. The cerebral hemispheres and attached mid-brain were rapidly weighed on a torsion balance and homogenized in 9 volumes of cold buffer (6). The resulting suspension was diluted with an equal volume of cold buffer to give a final concentration of 5 per cent on a wet weight basis and pipetted immediately into the previously prepared Warburg reaction vessels. Substrates and inhibitors were freshly prepared as solutions in the buffer, and flask volumes were adjusted with buffer so that the final concentrations of inorganic salts were always the same as those indicated in the legend of figure 1. Preliminary experiments established the fact that the homogenization medium of choice (6) was the complete buffer solution. As was expected from previous reports (5, 7), omission of either magnesium or potassium from the reaction mixture diminished the rate of carbon dioxide output. A pH of 7.5 was arbitrarily adopted for these studies although the optimum may lie at higher values (7).²

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² Preliminary results of a study of the effect of carbon dioxide on brain cholinesterase activity show that the optimum pH is probably close to 7.5 in the system described here.

Figure 1 shows the effect of adding various amounts of tissue to the system. The rate of CO_2 output is a linear function of tissue concentration up to 50 mg. of tissue per flask. The rate falls off at higher tissue concentrations probably because substrate concentration is no longer near the optimum and retention of CO_2 by the suspension, for which no correction was made, becomes large enough to affect seriously the results. Determinations of activity were always made by using tissue concentrations within the linear portion of the curve.

Figure 2 A shows the effect on rate of increasing substrate concentration with the use of acetylcholine and acetyl β -methylcholine. The data show an optimum concentration of acetylcholine of $5 \times 10^{-3}\text{M}$ when 25 mg. tissue per flask are used. The optimum is shifted to higher concentrations when larger amounts of tissue are used; e.g. at 50 mg. tissue per flask substrate optimum becomes $1 \times 10^{-2}\text{M}$. At higher concentrations of acetylcholine there is an inhibition of the rate. This is not true of

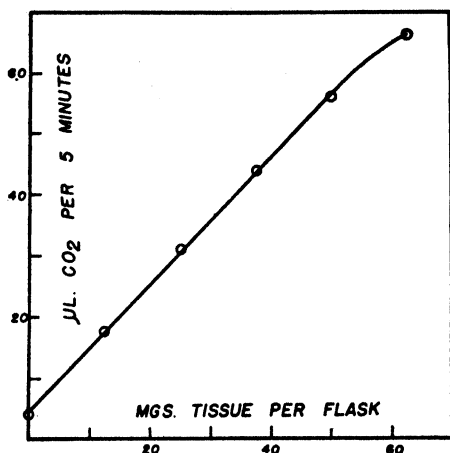


Fig. 1. EFFECT OF TISSUE CONCENTRATION on the rate of CO_2 evolution. Total volume 2.2 ml. containing final concentrations was as follows: 0.025M NaHCO_3 , 0.075M KCl , 0.075M NaCl , 0.04M MgCl_2 , 0.01M acetylcholine-bromide (added from sidearm after 15-min. equilibration period), 5 per cent CO_2 , 95 per cent N_2 in gas phase, temperature 37.5°C . Tissue was added as a 5 per cent homogenate. Each point is an average of 10 determinations. The curve for each experiment was linear up to 50 mg. tissue per flask. Microliters carbon dioxide per 5 minutes is calculated on basis of first 20 minutes during which period rate is linear.

acetyl β -methylcholine where the rate curve reaches a maximum and then plateaus. We have used acetylcholine as a substrate since this is presumably the physiologically occurring compound, and attempted to work at or near the optimum concentration. Figures 2 B, C, and D present an analysis according to Lineweaver and Burk's Case III (8) of a typical experiment on acetylcholine concentration. Similar treatment of the data from 5 experiments gave values of n , which is evaluated by the slope of curve in figure 2 C, ranging from 1.7 to 2.2. When 2.0 is substituted for n in the rate equation shown in figure 2 the experimental data fits a straight line (fig. 2 D), which justifies the previous evaluation. K_s was found to range from 1.66 to 2.60×10^{-3} with an average value of 2.03×10^{-3} and K_2 ranged from 1.40 to 1.81×10^{-2} with an average value of 1.64×10^{-2} .

*Effect of β -chlorinated Amines.*³ The degree of inhibition by the β -chlorinated

³ These amines were obtained through the courtesy of the University of Chicago Toxicity Laboratory.

amines is influenced by the time of incubation of the inhibitor with the enzyme before the addition of the substrate. This point is illustrated by the curves in figure 3, which show that the maximum inhibition is reached at about 30 minutes. Therefore, the standard procedure adopted is the practice of incubation of the enzyme with inhibitor for 40 minutes before the addition of the substrate. The equilibrium of enzyme with substrate appears to be practically instantaneous at optimum substrate concen-

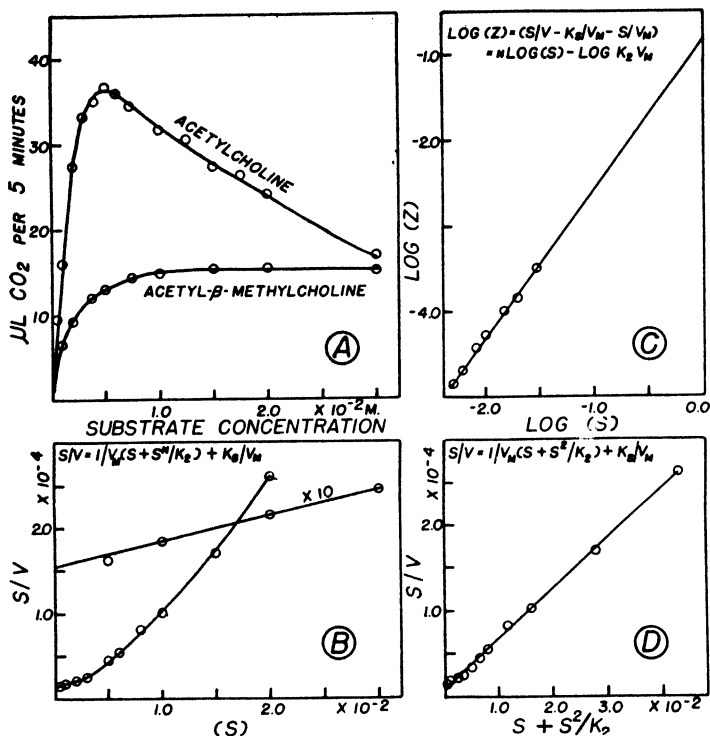


Fig. 2. EFFECT OF SUBSTRATE CONCENTRATION on brain cholinesterase activity. Inorganic salts, temperature and gas phase were the same as those in figure 1; 25 mg. tissue per flask. Microliters of carbon dioxide per 5 minutes were calculated on basis of first 20 minutes except at low substrate concentrations where first 10 minutes were used. Curves B, C and D analyze curve A according to Lineweaver and Burk's Case III (8). Slope of curve C = 1.8.

trations because no increase in rate appears over an hour's hydrolysis and the rate remains almost constant for long periods provided additional substrate is added from time to time to maintain optimum concentrations.

Table 1 is a summary of the inhibitory effect of the β -chlorinated amines. Those compounds which inhibited caused a 50 per cent decrease in enzyme activity over a relatively narrow range of concentration, 0.25 to $4.5 \times 10^{-4} \text{ M}$. The non-inhibitors had no effect on enzyme activity at these concentrations or higher.

A typical inhibition curve over a wide range of concentration is given for one of the amines in figure 4 and all the other inhibitory nitrogen mustards have the same

type of curve shifted to the right or left depending upon the potency of the compound. The inhibition approaches zero asymptotically at the lowest concentrations but there is an anomalous flattening at high concentrations where the inhibition should approach 100 per cent in the same manner. This flattening of the inhibition curve may indicate the presence of another enzyme which hydrolyzes the substrate but is not inhibited by this group of compounds.

Although the data in table 1 and figure 4 were obtained by using acetylcholine as a substrate, the β -chlorinated amines inhibit the hydrolysis of acetyl β -methylcholine to about the same degree. For example, $2.5 \times 10^{-4}M$ butyl bis(β -chloroethyl)-amine inhibits brain cholinesterase 50 per cent when the methylated ester is used as substrate.

Inhibition of cholinesterase activity by the β -chlorinated amines can be completely reversed by addition of cysteine to the system. Figure 5 shows that the reversal is complete when the ratio of inhibitor to cysteine concentration is 1:3. This

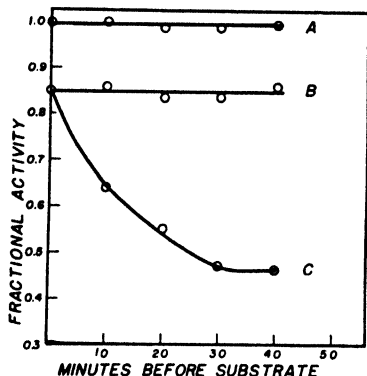


Fig. 3. EFFECT OF INCUBATION of enzyme with inhibitor before addition of substrate. Reaction mixture and conditions are the same as those in figure 1. Curve A = no inhibitor, substrate added at times indicated. Curve B = butyl bis(β -chloroethyl)amine and substrate added at times indicated. Curve C = butyl bis(β -chloroethyl)amine added at zero time, substrate added at times indicated.

reversal can be demonstrated only if the cysteine is added to the enzyme before or simultaneously with the inhibitor. If the inhibitor is incubated first with the enzyme and the cysteine added later very little reversal is observed.

Attempts to determine the type of inhibition, competitive or non-competitive, by the β -chlorinated amines using the methods described by Ebersole *et al.* (9) lead us to believe that the inhibition is non-competitive, probably of type III (9); but despite many attempts we have been unable to obtain conclusive data on this point.

Other Convulsants and Anticonvulsants. Table 2 presents the results from the use of some of the standard convulsants and anticonvulsants. Strychnine and nicotine are both inhibitors of brain cholinesterase, but metrazol and picrotoxin are not. Neither strychnine nor nicotine inhibition was reversed by cysteine. None of the 5 anticonvulsants studied inhibited cholinesterase nor did any of these anticonvulsants reverse the inhibitory effects of any of the compounds used in this study.

DISCUSSION

Inhibition of certain cholinesterases by high concentrations of acetylcholine has long been known, but as late as 1946 Bodansky (7) declined to evaluate the effect

because of "very few and incomplete data in the literature." However, Wright and Sabine (10) have recently published a very thorough study on the inhibition by high concentrations of acetylcholine of the human erythrocyte cholinesterase and Augustinsson (11) has also reported very extensive data in this regard on a number of cholinesterases. Figure 2 shows that the brain cholinesterase system studied here fits the formulation of Lineweaver and Burk's *case III* (8):

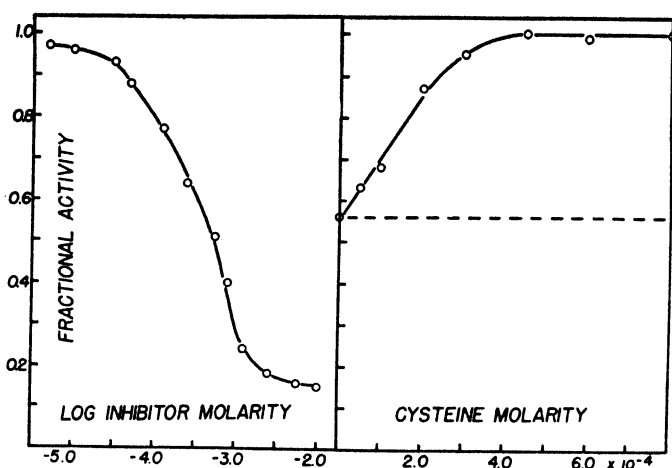
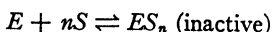
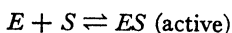


Fig. 4 (left). INHIBITION OF BRAIN CHOLINESTERASE by butyl bis(β -chloroethyl)amine. Reaction mixture and conditions are the same as those in table I. All points are averages of 3 experiments agreeing within ± 5 per cent.

Fig. 5 (right). REVERSAL BY CYSTEINE of β -chlorinated amine inhibition of brain cholinesterase. Reaction mixture and conditions are the same as those in figure 1. Inhibitor and cysteine were added to enzyme simultaneously 40 minutes before addition of substrate. 1.8×10^{-4} M butyl bis(β -chloroethyl)amine was used as inhibitor. Broken line indicates activity level without addition of cysteine

n is evaluated by the slope of curve in figure 2 C and shown to be 2.0 which is the same value as that found for the erythrocyte enzyme (10) and assumed by Augustinsson (11). The value, $K_2 = 1.6 \times 10^{-2}$, found here for the brain enzyme is nearly the same as that for the erythrocyte enzyme, $K_2 = 1.5 \times 10^{-2}$ (10). It is interesting to note that the addition of a methyl group to acetylcholine is enough change to prevent the combination of two molecules of the substrate with the active center of an enzyme molecule and the consequent formation of an inactive complex.

The correlation is good between the brain-cholinesterase inhibiting ability and the convulsant activity of the β -chlorinated amines. All of the compounds in *groups B* and *C*, table 1, are convulsant (1) and are also inhibitors of brain cholinesterase. All of the compounds in *group A*, table 1, are not convulsant (1) and are not inhibitors. It appears that the same structural requirements which were found for convulsant

activity in this group of compounds (1) (namely, that the minimum necessary structure is either $\text{CH}_3\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$ or $\text{CH}_3\text{N}(\text{C}_2\text{H}_5)\text{C}_2\text{H}_3\text{Cl}_2$ are also those necessary for brain-cholinesterase inhibitory activity. In the case of the compounds listed in *group C*, table 1, it was found that the relatively large blocking moieties (12), benzyl, diphenyl and naphthyl, can substitute for one of the chlorine groups as far as cholinesterase inhibiting ability is concerned, but this group of compounds are less potent convulsants than those in *group B*, table 1 (1).

The correlation between structure and activity just discussed appears to be related to the ability of the active compounds to form quaternary ammonium com-

TABLE 1. EFFECT OF β -CHLORINATED AMINES ON ACTIVITY OF BRAIN CHOLINESTERASE

<i>Group A (non-inhibitors)</i>	<i>Highest Molarity Tested</i>
Bis(β -chloroethyl)amine	1.3×10^{-8}
Methylethyl(β -chloroethyl)amine	8.0×10^{-4}
Dimethyl(β -dichloroisopropyl)amine	4.0
Methyl bis(β -cyanoethyl)amine	4.0
<i>Group B (inhibitors)</i>	<i>Molarity at 50% Inhibition</i>
Methyl bis(β -chloroethyl)amine	1.2×10^{-4}
Ethyl "	1.8
Propyl "	2.0
Butyl "	4.5
Methoxy "	4.0
Benzyl "	0.3
Diethyl(β -dichloroisopropyl)amine	3.0
Diethyl(β -dichloroisobutyl)amine	2.6
<i>Group C (inhibitors)</i>	<i>Molarity at 50% Inhibition</i>
N-(2-(2-biphenyloxy)ethyl)-N-(2-chloroethyl)butylamine	2.5×10^{-6}
N-(2-bromoethyl)-N-ethyl-1-naphthalenemethylamine	3.0×10^{-6}
N-(2-chloroethyl)dibenzylamine	3.0×10^{-4}

Reaction mixture and conditions are the same as those indicated in figure 1. Inhibitor incubated with enzyme 40 minutes before addition of substrate. Each value average of at least 3 determinations which agreed within ± 5 per cent. All compounds in *group A* were not convulsant in cats at doses of 100 mg/kg., i.v.; all compounds in *group B* were convulsant in cats at doses less than 10 mg/kg., i.v.; compounds in *group C* were convulsant in cats when potentiated by CO_2 inhalation at doses less than 25 mg/kg., i.v. (1).

pounds in solution (13). There is the additional requirement that after the formation of the quaternary compound there must be a residual chlorine or a large blocking moiety (12) on the active compound. Sanz reports in reference (11) that quaternary ammonium bases are powerful inhibitors of 'specific' cholinesterase (the brain enzyme is of this type, 11) while tertiary amines are more powerful inhibitors of the 'non-specific' type.

Cysteine reversal of inhibition by the nitrogen mustards is particularly interesting in view of the fact that cholinesterase is an enzyme, the activity of which has been reported to depend upon the presence of free -SH groups (14). The reversal may be caused by the regeneration or protection of active groupings or it may be caused

by direct action upon the inhibitor by the cysteine similar to the thiosulfate protection observed by Barron *et al.* (2), but in that instance 100 times as much thiosulfate as amine was required for reversal whereas here only 3 times as much cysteine as amine was completely effective.

The results obtained with other convulsants, strychnine, nicotine, metrazol, and picrotoxin, agree with those obtained by other workers (11). It is apparent that here the correlation between cholinesterase inhibiting ability and convulsant activity breaks down, since 2 of the convulsants were inhibitory and 2 were not, and an examination of the summary of inhibitors given by Augustinsson (11) will show that convulsants may be found, other than the ones studied here, some of which inhibit and others of which do not inhibit cholinesterase. If the physiological effects of the nitrogen mustards, eserine and diisopropylfluorophosphate are to be attributed in a large measure to their ability to inhibit cholinesterase as has been proposed (3, 15), and if this is the mechanism whereby they produce convulsions, then we must assume that there are at least 2 ways in which chemical convulsants can act in order to explain

TABLE 2. EFFECT OF OTHER CONVULSANTS AND ANTICONVULSANTS ON BRAIN CHOLINESTERASE¹

GROUP A (INHIBITORS)	MOLARITY AT 50% INHIBITION	GROUP B (NON-INHIBITORS)	HIGHEST MOLARITY TESTED
Strychnine	1.6×10^{-4}	Metrazol	1.0×10^{-3}
Nicotine	5.0×10^{-3}	Picrotoxin	1.0
		Phenobarbital	1.0
		Trimethadione	1.0
		Phenacetylurea	1.0
		Diphenylhydantoin	1.0
		Atropine	8.0×10^{-4}

¹ Reaction mixture and conditions same as table 1.

the lack of correlation just discussed. There is evidence from another source that convulsants differ in their point of attack in that some of them are potentiated by inhalation of CO₂ and others are inhibited by this procedure (1). It is hoped that further work will show a correlation between groups of convulsants selected by these criteria and by their cholinesterase inhibiting ability. Such a relationship would be particularly valuable because of the fact that grand mal and petit mal show opposite reactions to the inhalation of CO₂ (16).

Failure to find any relationship between the anticonvulsants and the cholinesterase system, either as inhibitors or reversers of inhibitors, was not unexpected since *in vivo* reversal has been demonstrated in only a few cases and then with certain experimental qualifications (1, 17).

SUMMARY

A study was undertaken of the inhibition of the cholinesterase system of rat brain by excess substrate, a series of β -chlorinated amines, and some other convulsant and anticonvulsant drugs. The enzyme was shown to combine with 2 moles of acetylcholine to form an inactive complex with a dissociation constant of $1.6 \times$

10^{-2} M. The structure necessary for inhibitory activity by the β -chlorinated amines was elucidated and shown to correlate with that necessary for convulsant activity, in both cases the minimum necessary structure for activity being $\text{CH}_3\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$ or $\text{CH}_3\text{N}(\text{C}_2\text{H}_5)\text{C}_2\text{H}_4\text{Cl}_2$. Strychnine and nicotine were found to be inhibitors of cholinesterase, but metrazol and picrotoxin were not inhibitors. The anticonvulsants, phenobarbital, trimethadione, phenacetylurea, diphenylhydantoin, and atropine had no effect on the enzyme, nor did they reverse the effect of any of the inhibitors studied. Inhibition of the enzyme by the β -chlorinated amines was reversed by cysteine. The results are discussed with regard to the mechanism of action of convulsant drugs.

The author wishes to thank Miss Ruth Hurwitz and Mr. Richard C. Wang for valuable technical assistance.

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CONVULSIONS IN CEREBELLUM AND CEREBRUM INDUCED BY β -CHLORINATED AMINES¹

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THE inhalation of carbon dioxide in oxygen antagonizes convulsions induced by electric shock (1), metrazol (1-3), strychnine (2-4) and insulin (5, 6). Conversely, it synergizes those caused by the ingestion of certain proteins treated with nitrogen trichloride (7), DDT (8), and high oxygen pressures (9). Moreover, it is known that high concentrations of CO₂ raise the threshold of nerve (10), inhibit petit mal (11), and are therapeutic in certain mental states (12). To evaluate these actions of CO₂ further, various convulsant agents were administered before or after its inhalation and the effects studied. This paper presents the results obtained with the use of certain β -chlorinated amines and allied compounds.

The β -chlorinated amines, nitrogen mustards, have been extensively investigated in recent times. The salient features of their properties have been reviewed (13). It occurred to the authors that an investigation of convulsant action of these amines would be profitable and might provide valuable information in elucidating convulsant mechanisms. A further reason for a thorough investigation was the discovery early in this study that inhalation of gas mixtures high in CO₂ had a potentiating effect on nitrogen mustard convulsions.

METHODS

Normal cats weighing from 1.5 to 3.0 kg. were used throughout. Under divinyl ether anesthesia, a tracheal cannula was introduced, the femoral veins isolated, and the cranium, including the area over the cerebellum, exposed. Divinyl anesthesia was then discontinued and paralysis induced with 10-mg/kg. dihydro- β -erythroidine². A continuous intravenous drip of 0.9 per cent saline containing 0.5 mg/cc. erythroidine was introduced into one femoral vein and adjusted to 0.5 cc/min. Respiration was maintained artificially through a Palmer respirator designed to allow adjustment of stroke volume and rate and the introduction of any desired gas mixture. Screw electrodes were placed bilaterally, 2 over the cerebellum and 2 over the parietal cortex. Bipolar and monopolar (reference electrode on tongue) recordings of the electrical activity of heart and brain were obtained on a Grass 6-channel electroencephalograph.

Convulsants were injected into the femoral vein as freshly prepared aqueous solutions of their hydrochloride unless otherwise indicated. Concentrations were adjusted so that not less than 0.5 cc. and not more than 2.0 cc. were injected at any one time.

Gas mixtures containing 10 to 30 per cent CO₂ plus O₂ were obtained commercially or prepared in this laboratory. Mixtures were analyzed using a Van Slyke apparatus, and were used only when they were within \pm 0.5 per cent of their nominal values.

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² Obtained through the courtesy of Merck & Co., Inc., Rahway, N. J.

RESULTS

Infusion of dihydro- β -erythroidine has only minimal effects on the EEG of the cat and has been used in this laboratory for a number of years to eliminate undesirable muscle potentials from brain wave recordings in animals³. Inhalation of gas mixtures containing high concentrations of CO₂ results in the appearance in the EEG of considerable high-frequency, low-amplitude waves, but other than this there is little change in the over-all picture and certainly nothing which would be confused with seizure activity (1).

Data obtained from a survey of 25 nitrogen mustards are summarized in table 1. It will be seen that the nitrogen mustards are convulsant in the range from 2.0 to 3.0 mg/kg., in the presence of high (30%) concentrations of CO₂, and for any given nitrogen mustard the convulsant dose with CO₂ is approximately 50 per cent of the normal convulsant dose. Furthermore, without CO₂ the period of lag between injection and start of convulsions is from 10 to 15 minutes while with CO₂ the lag period is always less than 5 minutes and is usually about 90 seconds. These compounds are potent convulsants compared to penta methylene tetrazol (10 to 15 mg/kg.) or bromocamphor (18 mg/kg.) and are of about the same order of potency as strychnine (2.0-5.0 mg/kg.).

Preliminary experimentation with various concentrations of CO₂ and various periods of inhalation of the gas mixtures showed that the potentiating effect of CO₂ could be elicited by concentrations as low as 10 per cent CO₂ and 90 per cent O₂ if applied for a sufficiently long time. The period of inhalation necessary to potentiate convulsions was roughly in inverse proportion to the concentration of CO₂ in the mixture. Occasionally, seizures were not seen while the CO₂ was being administered, but developed immediately after the animal was returned to room air. Results reported in table 1 were obtained with 30 per cent CO₂ and 70 per cent O₂ breathed for 3 minutes, the CO₂ being continued the full time even though a seizure occurred during its administration.

With doses of nitrogen mustard just above the convulsive level with CO₂, the spiking and seizure activity almost always appeared first in the leads over the cerebellum (fig. 1). In some cases, it was possible to obtain records in which the seizure was limited exclusively to the cerebellar leads (fig. 2). With higher doses, the seizure activity appeared first in the cerebellar leads, but was also quickly seen in the cerebral leads.

β -chloroethylmorpholine was convulsant at high (75 mg/kg.) doses, but not potentiated by CO₂; 4-nitrosomorpholine was not convulsive at 225 mg/kg. Mustard gas, bis- β -chloroethyl sulphide, was not convulsive at the highest dose tolerated, 25 mg/kg. A phosphorus analog, compound 25, was not convulsive, Dibenamine was weakly convulsant at approximately 25 mg/kg., but only with CO₂. Phenobarbital, diphenylhydantoin or trimethadione were not effective in antagonizing the convulsant action of the nitrogen mustards⁴.

³ Unpublished results.

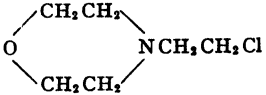
⁴ The authors thank Dr. Graham Chen for performing many of the experiments testing the effect of anticonvulsants.

It should be mentioned here that because of the limited number of animals per compound and the injection techniques employed, the convulsant doses listed are

TABLE 1. CONVULSANT ACTIVITY OF β -CHLORINATED AMINES¹

NO.	COMPOUND	FORMULA	CONVULSIVE DOSE (MG./KG.)	
			With CO ₂ ^a	Without CO ₂
1	Bis(β -chloroethyl) amine	$\text{HN}(\text{C}_2\text{H}_4\text{Cl})_2$	700 No convulsion	
2	Methyl bis(β -chloroethyl)amine	$\text{CH}_3\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	5.5 <i>cb</i> ^a 10.0 <i>cb, cx</i>	25 <i>cb, cx</i>
3	Ethyl bis(β -chloroethyl)amine	$\text{C}_2\text{H}_5\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	7.3 <i>cb</i> 10.0 <i>cb, cx</i>	22 <i>cb, cx</i>
4	N-propyl bis(β -chloroethyl)amine	$\text{C}_3\text{H}_7\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	1.0 <i>cb</i> 4.6 <i>status</i>	7.0 <i>cb, cx</i>
5	N-butyl bis(β -chloroethyl)amine	$\text{C}_4\text{H}_9\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	2.9 <i>status</i> ^a 2.0 <i>cb</i> 3.0 <i>status</i>	5.5 <i>cb</i> 5.0 <i>cb</i> 8.5 <i>cb, cx</i> 7.5 <i>cb, cx</i> 7.5 <i>cb, cx</i>
6	Tris(β -chloroethyl) amine	$\text{N}(\text{C}_2\text{H}_4\text{Cl})_3$	12.5 <i>status</i> 2.5 <i>cb</i> 11.7 <i>status</i>	4.5 <i>cb, cx</i>
7	Diethyl 2, 2-dichloroisobutylamine	$(\text{C}_2\text{H}_5)_2\text{NCH}_2(\text{CH}_3)\text{CCl}_2\text{CH}_3$	2.3 <i>cl, cx</i>	6.0 <i>cb, cx</i>
8	Benzyl bis(β -chloroethyl)amine	$\text{C}_6\text{H}_5\text{CH}_2\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	2.5 <i>cb, cx</i> 4.1 <i>status</i>	4.5 <i>status</i>
9	Diethyl 2, 2-dichloroiso-propylamine	$(\text{C}_2\text{H}_5)_2\text{NCH}(\text{CH}_3)\text{CHCl}_2$	1.7 <i>cb</i>	4.0 <i>cb, cx</i>
10	Tetrakis(β -chloroethyl) ethylenediamine	$(\text{C}_2\text{H}_4\text{Cl})_2\text{NCH}_2\text{CH}_2\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	2.4 <i>cb, cx</i>	
11	Allyl bis(β -chloroethyl) amine	$(\text{C}_2\text{H}_4\text{Cl})_2\text{NCH}_2\text{CH}:\text{CH}_2$	3.1 <i>cb, cx</i>	5.4 <i>cb, cx</i>
12	Methoxyethyl bis (β -chloroethyl)amine	$(\text{C}_2\text{H}_4\text{Cl})_2\text{NCH}_2\text{CH}_2\text{OCH}_3$	2.9 <i>status</i>	
13	Methylethyl β -chloroethylamine	$\text{CH}_3\text{N}(\text{C}_2\text{H}_5)\text{C}_2\text{H}_4\text{Cl}$	81 No convulsion 75 No convulsion	
14	Dimethyl β -chloroethyl amine	$(\text{CH}_3)_2\text{NC}_2\text{H}_4\text{Cl}$	50 No convulsion	
15	Trichloromethyl bis (β -chloroethyl) amine	$\text{CCl}_3\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$	30 No convulsion	
16	Dimethyl 2, 2-chloropropyl amine	$(\text{CH}_3)_2\text{NCH}_2\text{CCl}_2\text{CH}_3$	118 No convulsion	
17	Triethanolamine	$\text{N}(\text{C}_2\text{H}_4\text{OH})_3$	176 No convulsion	
18	Methyl bis(cyanoethyl) amine	$\text{CH}_3\text{N}(\text{C}_2\text{H}_4\text{CN})_2$	57 No convulsion	

TABLE 1—Continued

NO.	COMPOUND	FORMULA	CONVULSIVE DOSE (MG/KG.)	
			With CO ₂ ^a	Without CO ₂
19	Methyl bis(thiocyanatoethyl) amine	CH ₃ N(C ₂ H ₄ SCN) ₂	10 No convulsion, cortex iso-electric, blood gray	
20	β-chloroethylmorpholine		90 One seizure	75 One seizure
21	4-Nitrosomorpholine		225 No convulsion	
22	Bis(β-chloroethyl) sulfide	S(C ₂ H ₄ Cl) ₂	25 No convulsion, cortex iso-electric	
23	Bis 2, 2-chloroethyl-dimethyl chloride	(CH ₃) ₂ (C ₂ H ₄ Cl) ₂ NCl	178 No convulsion	
24	Dibenzyl β-chloroethylamine 'Di-benamine'	(C ₆ H ₅ CH ₂) ₂ NC ₂ H ₄ Cl	10 One convulsion 28 No convulsion	110 No convulsion
25	Tris(chloromethyl) phosphine	P(CH ₂ Cl) ₃	18 <i>cb, cx</i> 206 No convulsion	

¹ The majority of the β-chlorinated amines were obtained through the courtesy of the University of Chicago Toxicity Laboratory.

² Thirty per cent CO₂ and 70 per cent O₂ mixture administered for 3 minutes (see text).

³ *cb, cx*, or *status* indicate that the seizure appeared in the cerebellum (*cb*), cerebrum (parietal cortex) (*cx*) or both, or that the animal went into *status*.

⁴ A horizontal line separating sets of figures indicates that the dosages were determined on separate animals. When no separation is indicated, the dosages were determined on the same animal, with a one-hour recovery period after the end of the seizures induced by the first dose. Dosages with and without CO₂ were determined on separate animals. Forty-five cats were used to obtain these data.

probably uncertain within ± 0.5 mg/kg. However, in the case of *compound 7* (table 1) which was tested on 7 animals the convulsant doses were quite consistent. Compounds were considered non-convulsive if no abnormalities in the EEG were observed up to 50 mg/kg. In point of fact, all the nitrogen mustards which were convulsive were active at doses below 25 mg/kg., even without CO₂ inhalation.

DISCUSSION

If the general structure of the nitrogen mustards be represented by R_1-N-R_2 ,



then when R_2 and R_3 are $-\text{CH}_2\text{CH}_2\text{Cl}$, as R_1 is increased from methyl to butyl there is a slight increase in toxicity. When R_1 is also $-\text{CH}_2\text{CH}_2\text{Cl}$, the compound is no more

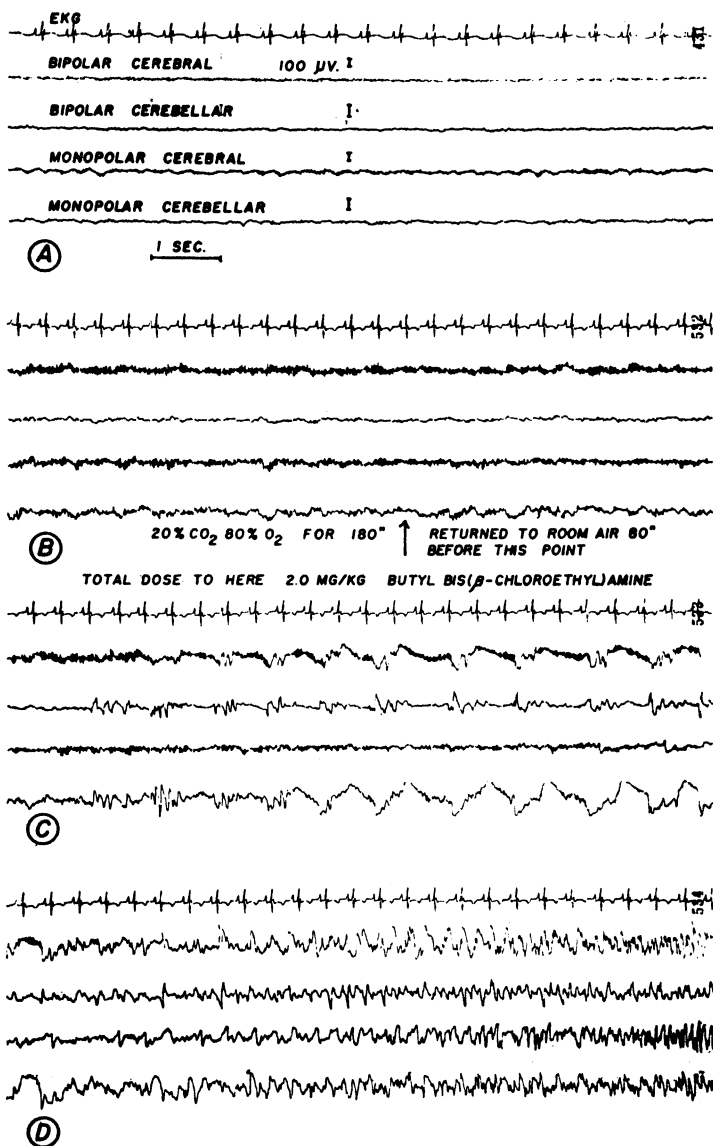


FIG. 1. SEIZURE ACTIVITY induced by Butyl bis(β -chloroethyl)amine and CO₂. *A* shows the appearance of the EEG before CO₂ or nitrogen mustard treatment. The gain is low to protect the pens during subsequent seizure. *B*, *C*, and *D* are consecutive and show the first appearance of seizure activity in the cerebellar cortex leads and subsequent appearance in the cerebral cortex leads.

toxic than when R_1 is butyl. With regard to the question of the structure necessary for convulsant activity, the following points may be deduced from the data. The

compound must be a tertiary amine, since *compound 1* is not convulsant and *compound 2* is. In the general structure just referred to, R_1 may be one of a variety of groups: methyl, ethyl, propyl, butyl, benzyl, allyl or methoxy. R_2 must be at least ethyl since *compound 16* is not convulsive and *compound 7* is. There must be at least

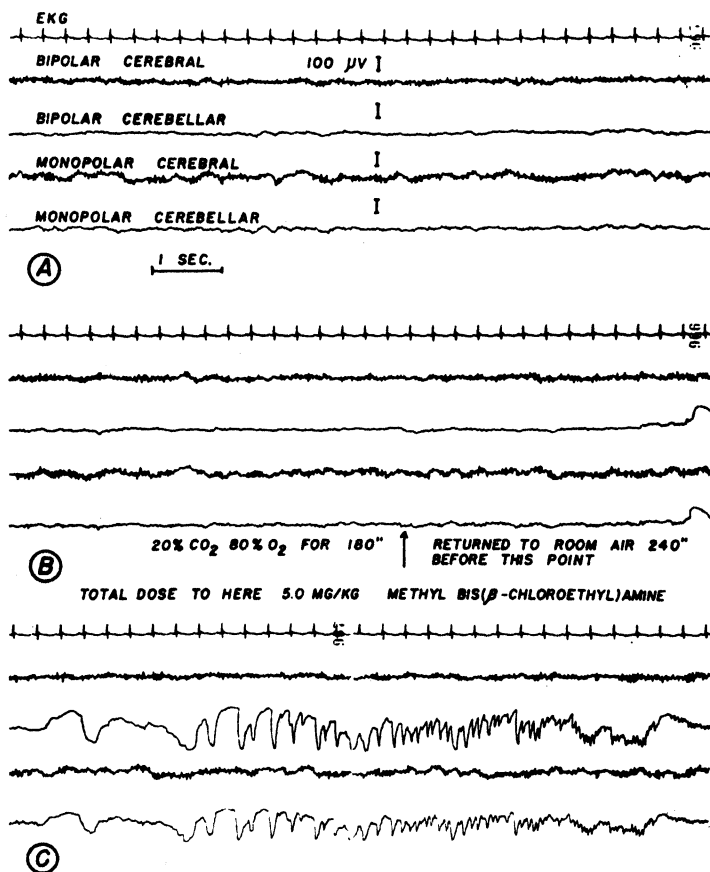


FIG. 2. SEIZURE ACTIVITY induced by Methyl bis(β -chloroethyl)amine and CO_2 . A is control record as in fig. 1. B and C are 5 seconds apart and show seizure activity confined to the cerebellar cortex leads and return to normal at the end of C without any seizure activity appearing in the cerebral cortex leads.

2 chlorine groups on the molecule, but both may be on one side-chain or one each on R_2 and R_3 . (Compare *compounds 2, 7, 9, 13*.) Whether or not chlorine may be replaced by other halogens could not be decided from the compounds at our disposal; but OH, CN, or SCN substituted for chlorine does not give an active compound. The halogen probably needs to be β - to the nitrogen (14), but this again could not be tested with our series. Thus it appears that in the minimum convulsive structure, R_1 must be at least methyl, R_2 must be at least ethyl, and R_3 must be at least ethyl and carry two β -chlor groups or if it carries only one, then R_2 must also carry a β -chlor group.

The nitrogen mustards and other β -chloroamines form ethylene-ammonium rings in aqueous solution (15) and it has been proposed that it is in this form that the compounds are physiologically active (13). In the present work those mustards which were active form, upon cyclization, a compound containing quarternary nitrogen; whereas *compound 1*, which was not active, yields only a tertiary amine (15). Since the cyclization involves loss of one chlorine atom, it may be that the requirement for at least 2 chlorine atoms in the active compound indicates the necessity for a residual chlorine after ring formation. Of course, in the bis- β -chloroethyl series, the reaction will progress until all of the chlorine is lost and the data herein presented are not adequate to show which of the intermediates is the active form. On the other hand, 'dibenamine' is convulsive at higher doses, but here R_1 and R_2 are both relatively large blocking moieties (16) and may substitute for the postulated residual chlorine.

Convulsions, incoordination and tremors induced by parenteral administration of various nitrogen mustards in mice, rats, rabbits, horses and dogs have previously been reported (17-19). Hecht and Anderson report convulsions in man after the injection of dibenamine (20). This same effect had been noted previously in cats and mice by Nickerson and Goodman (21). Goldin *et al.* (22, 23) noted that β -chloroethylmorpholine induced a specific neurological effect characterized by general hyperactivity and incoordination in mice, rats, cats and dogs. This drug also produced extreme ataxia, incoordination and hyperactivity in a monkey (23). Examinations of mice in their studies showed early scattered ganglion cell changes, particularly in the Purkinje cells. Goldin, *et al.* (24) found a series of nitrogen mustard analogs which produced hyperactivity, incoordination, and choreic head movements in mice. Again lesions were found in the cerebellum and brain stem. It is of interest to note that intravenous or subcutaneous injection of methyl bis (β -chloroethyl) amine gave rise to kinetic tremor, incoordination, paresis, convulsions, and hypotonia in various animals, but when the drug was administered orally no neurotoxic signs were observed (19).

There is a growing body of evidence to show that convulsants may be divided into two groups: one potentiated, the other inhibited by CO_2 . The nitrogen mustards belong in the first group together with azenized proteins (7), DDT (8) and high pressures of O_2 (9). Metrazol, electric shock, strychnine and insulin fall in the second group (1-6). The way in which the CO_2 works is unknown. However, it is noted that with convulsants antagonized by CO_2 the convulsion starts in the cerebrum, while with those which are potentiated it is first seen in the cerebellum. Seizures may originate in a deeper structure and the activity recorded at the surface may only reflect a preferential pathway from the active center to the cerebellum. Nevertheless, this seizure in the cerebellum does serve to explain the observations of other workers on the gross behavior of animals under the influence of the β -chloroethylamines (17-24).

SUMMARY

The convulsant activity of a series of 25 β -chloroethylamines has been studied in cats and the necessary configuration for convulsant activity found to be $\text{CH}_3\text{N}(\text{C}_2\text{H}_4\text{Cl})_2$ or $\text{CH}_3\text{N}(\text{C}_2\text{H}_5)(\text{C}_2\text{H}_3\text{Cl}_2)$. The threshold dose of the compounds is approximately halved and the time of onset of seizures greatly reduced by inhalation of gas

mixtures containing 15 to 30 per cent CO₂. Seizure discharge was first observed in the cerebellar leads.

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EFFECT OF STIMULATION ON THE PHOSPHATE ESTERS OF THE BRAIN

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THE energy requirements of the brain are supplied mainly by glucose, a part of which is oxidized to carbon dioxide and a part is glycolyzed to form lactic acid. It is believed that the energy derived from the breakdown of glucose is made available for the synthetic processes of the cell by the phosphate esters, which thereby play a central part in the brain metabolism. The 'high-energy' phosphate esters would therefore act as a reserve of energy available for the synthesis of acetylcholine and for other processes involved in the functional activity of the brain. In agreement with this view it has been shown that the fall in the acetylcholine content of the brain in electrically induced convulsions (1) is associated with a fall in phosphocreatine and a rise in the inorganic phosphate and lactic acid (2, 3).

The present investigation was carried out to obtain further information about the dynamic aspects of the changes in the brain *in vivo*. The sequence of changes in the rat brain after stimulation was studied by determining the phosphate esters and inorganic phosphate in brains of animals killed at different times during and after electrical stimulation. In this way evidence of the rate of breakdown and resynthesis of phosphocreatine under these conditions was obtained. Further experiments were carried out to study the changes in the phosphate esters of the brain under normal physiological conditions.

METHODS

Young Wistar albino rats weighing 25 to 40 gm. were killed by immersion in liquid air, which produced a rapid fixation of any biochemical changes in the tissues and limited postmortem glycolysis. Electrical stimulation of the brain was carried out with stainless steel electrodes of 0.25-cm.² area, which were applied to the scalp 0.5 cm. posterior to the eyes. Contact was obtained by previously cutting the fur in this region with sharp scissors and applying electrode jelly. The current used was 50 cycles A.C. at 40 volts. Stimulation for 5 seconds produced an immediate generalized muscular spasm, followed by relaxation due to the concussion. Convulsions started about 7 seconds after cessation of the stimulation and continued for about 60 seconds; they were severe at the start, but gradually became less severe and more intermittent towards the end of this period. The procedure was similar to that used in the 'electroshock' treatment of psychiatric patients. The method used for producing emotional excitement was similar to that described in previous papers, where other experimental details have also been given (1, 4-6). Littermates were taken for comparison of normal and emotionally excited animals.

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Separation of Phosphate Esters. Determination of the various acid-soluble phosphorus compounds in the brain depended on a simple fractionation of the calcium salts by a method similar to that of Stone (2). By this procedure the phosphorus compounds were separated into 4 fractions containing 1) inorganic phosphate, 2) ATP and ADP (adenosine tri- and di-phosphate), 3) phosphocreatine (phosphagen) and 4) the esters including hexose phosphates contained in the residual or calcium-soluble fraction. This last fraction is referred to in the present paper as the 'hexose phosphate fraction.'

After fixation in liquid air, the brain (about 0.9 gm.) was removed and any particles of bone were carefully washed off with liquid air. The brain was rapidly weighed on a torsion balance and ground with 9 ml. 5 per cent trichloroacetic acid at -5°C . in a cooled mortar. Rapid grinding was essential as the temperature coefficient of adenosine triphosphatase is low and the enzyme has appreciable activity even at 0°C . The suspension was allowed to stand for 10 minutes at -5°C ., centrifuged, and a 6-ml. aliquot of the supernatant solution was treated with 1-ml. calcium chloride solution (50% w/w $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$). Absolute alcohol (1 ml. containing 0.1 mg. phenolphthalein) was added and the solution was cautiously adjusted to pH 8.2 by addition of 0.5 ml. 3N NaOH followed by smaller amounts of 0.1N NaOH. A further 0.15 ml. of 0.1N NaOH was then added and the solution was brought to 10 ml. with distilled water. Throughout this and subsequent procedures, the solution and reagents were kept at 0°C . to minimize the hydrolysis of phosphocreatine. A blank and two experimental tubes only were run in each series, to minimize the time of handling and therefore the loss of unstable phosphate esters by hydrolysis.

Kerr (7) showed that the calcium salt of ATP is only about 80 per cent precipitated at pH 8.2 and Stone (2) used a slight excess of calcium hydroxide, which he found increased the fraction precipitated. Lohmann (8) observed that ATP is appreciably hydrolyzed at an alkaline pH , but under the conditions chosen for the present work the loss of ATP was small, as the excess of alkali was slight, the temperature low and the handling brief. In 3 successive experiments the loss of ATP was equivalent to 0.5, 0.4 and 0.5 mg. P. per cent. This correction was therefore applied to the ATP and inorganic phosphate figures.

The suspension of soluble and insoluble calcium salts was centrifuged after standing for 20 minutes. The precipitate was washed by shaking once with 4 ml. of an alkaline solution of 10 per cent calcium trichloroacetate in 10 per cent alcohol and then recentrifuged. The precipitate was dissolved with 0.2 ml. 2N HCl and diluted to 6 ml. with water. Inorganic phosphate was determined in an aliquot of 2.5 ml. by the method of Fiske and Subbarow (9), using a molybdate reagent made with hydrochloric acid instead of sulfuric acid. The extinction coefficient was read after 10 minutes on a photoelectric colorimeter, with the use of an Ilford 204 red filter. Inorganic phosphate was determined in a further aliquot of 2.0 ml. after hydrolyzing with N HCl for 10 minutes at 100°C . to hydrolyze the ATP and ADP. As this solution was often slightly turbid a correction was applied by using a blank of similar turbidity. This was found more satisfactory than attempting to remove the turbidity by centrifuging while the color was developing, as recommended by Stone.

The original solution containing the soluble calcium salts was filtered and phos-

phocreatine was determined in an aliquot of 5 ml. by the method of Fiske and Subbarow (10). As the high salt concentration may cause a slight increase in color development, the standard phosphate was added to a blank tube containing a similar concentration of salts. Total phosphorus was determined in a 3-ml. aliquot after oxidation with 1 ml. 70 per cent perchloric acid by the method of King (11). Preliminary control experiments showed that under the conditions used no phosphorus was lost by volatilization and the color development was not affected by variation from 0.5N to 1N in the concentration of acid used. After dilution, the solution was heated on a waterbath at 100°C. for 10 minutes before estimating the phosphate.

The brain contains an appreciable quantity of silica (12), which is known to interfere with phosphate estimations. Control experiments showed that the estimations were not significantly affected by quantities up to 40 mg. per cent sodium silicate, which is more than is to be expected in the brain; but it was found advisable that the strength of the acid used in the color development should be approximately 0.6N and that the reading should be taken 10 minutes after adding the reducing agent.

RESULTS

Normal Series. The main object of the present investigation was to study changes in the high-energy phosphocreatine and adenosine polyphosphates. The extractable phosphorus compounds were therefore separated into 4 representative fractions and no attempt was made to follow the changes in every individual phosphate ester known to occur in the brain. The results are shown in table 1 for 1) phosphocreatine, 2) ATP and ADP, 3) inorganic phosphate, 4) the calcium-soluble or hexose phosphate fraction and 5) the total acid-soluble phosphorus determined in the brain. The rats included in the normal series were transferred to liquid air while in the normal waking state. Determinations were carried out under carefully standardized conditions and individual figures in each series showed a relatively small degree of scatter. In 10 consecutive experiments the individual phosphocreatine and adenosine phosphate figures showed a variation from the normal mean of less than 1.6 mg. P. per cent. The normal values agree with those previously reported by Le Page (13).

Effect of Electrical Stimulation. Electrical stimulation of the rat brain under the conditions described produced a rapid fall in the phosphocreatine content (fig. 1). Animals transferred to liquid air after 1 second of stimulation showed a loss of 50 per cent of the total brain phosphocreatine and over 70 per cent had disappeared in animals killed a few seconds later. The time taken for the transferral to liquid air was less than one second and the time of freezing was about 4 seconds for the cerebral cortex. Even if the fall continued at the same rate throughout the period of freezing, the rate of breakdown of phosphocreatine was rapid.

In animals killed during the period of electrical stimulation the fall in phosphocreatine (-6 mg. P. %) was accompanied by a small liberation of inorganic phosphate ($+2$ mg. P. %) and a significant increase in the hexose phosphate fraction ($+4$ mg. P. %). This would agree with a transfer of phosphate from phosphocreatine to glucose or glycogen, with a consequent increase in the hexose phosphate fraction, as the main change in the phosphate esters during the first few seconds of stimulation.

There was little evidence of any considerable breakdown of ATP although the level after stimulation was significantly lower than normal. If, as is generally believed, the phosphorylation of carbohydrate occurs through the transfer of phosphate from ATP, the rate of resynthesis of ATP from phosphocreatine must be extremely rapid.

During the preconvulsive period, before the commencement of convulsions, the animals were in a state of coma. The chief change in this period was a fall in the hexose phosphate fraction (-6 mg. P. per cent) with a corresponding liberation of inorganic phosphate ($+6$ mg. P. per cent). There was no significant resynthesis of

TABLE 1. PHOSPHATE ESTER CONTENT OF THE RAT BRAIN

RAT NO.	WT. OF RAT	CONDITION	INORG. P.	ATP AND ADP	PHOSPHO-CREATINE	HEXOSE P. FRACTION	TOTAL P.
	gm.		mg. P. %	mg. P. %	mg. P. %	mg. P. %	mg. P. %
1	35	Normal	14.0	13.4	10.7	15.8	53.9
2	35	"	17.3	15.8	9.6	11.6	54.3
3	40	"	14.3	16.2	10.0	18.3	58.8
4	30	"	13.8	13.3	8.2	21.1	56.4
5	25	"	11.1	15.5	11.2	18.9	56.7
6	30	"	13.9	16.2	11.2	17.3	58.6
7	25	"	11.9	14.5	9.5	16.1	52.0
8	30	"	12.9	15.0	8.2	19.1	55.2
9	32	"	13.7	13.5	10.0	15.0	52.2
10	35	"	14.9	15.5	9.7	15.0	55.1
MEAN.....			13.8	14.9	9.8	16.8	55.3
11	40	Anesthetized	7.9	11.9	16.4	25.2	61.8
12							
13							
14	40	"	14.6	16.3	12.1	17.6	60.5
15							
16	25	Sleeping	15.2	13.9	8.0	17.8	54.9
	25	"	9.9	13.1	11.7	17.3	52.0

Anesthesia was obtained by intraperitoneal injection of sodium pentobarbital, 50 mg/kg. Value for anesthetized rats are for 2 combined rat brains. The period of sleep was 15 to 30 minutes.

phosphocreatine and this may be caused by the fact that the tissues were anoxic, as respiration had ceased

The onset of convulsions was not marked by any sudden changes in the phosphate esters, such as occurs in the brain acetylcholine (1). The phosphocreatine remained low for some 10 seconds and then started to return to the normal level, even while intermittent convulsions were still in progress. At the same time there was a return to normal in the inorganic phosphate and ATP. It may be noted that the respiration recommenced at about the time the convulsions started, so that during this period the tissues were adequately supplied with glucose and oxygen. The rate of resynthesis of phosphocreatine under these conditions, calculated from the slope of the curve (fig. 1), was about 1.25 mg. phosphocreatine per gram fresh tissue per minute.

Changes under Physiological Conditions. Experiments with rats anesthetized

with sodium pentobarbital confirmed the increase during anesthesia in the phosphocreatine content of the brain, which was previously observed by Stone (2). Experiments with animals dropped into liquid air while asleep gave figures falling in the normal range (table 1).

In a series of 10 rats which had been excited for 4 to 7 minutes by rotation in a glass beaker there was no significant difference from the normal in the inorganic phosphate, ATP and ADP or hexose phosphate fractions, but the phosphocreatine

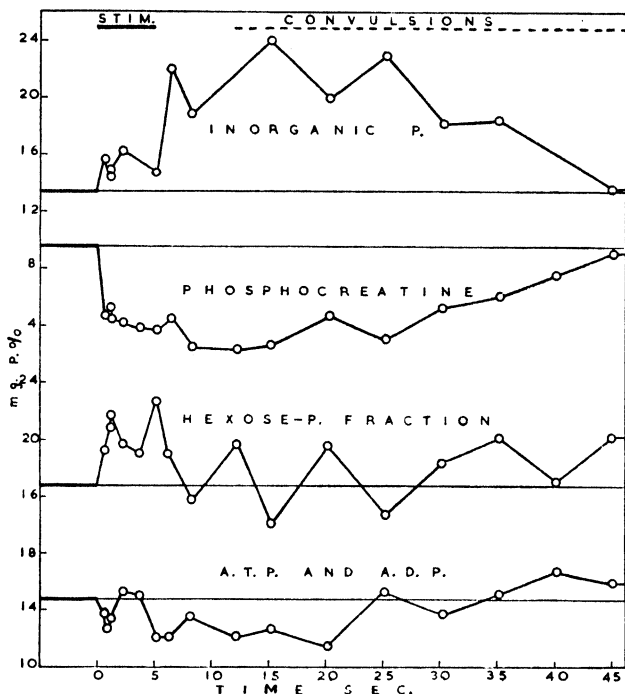


Fig. 1. EFFECT OF ELECTRICAL STIMULATION on the phosphate esters and inorganic phosphate of the rat brain. Normal mean levels are given by horizontal lines. Points give values for rats killed at the times indicated. Stimulation was continued for 5 seconds except for rats killed after a shorter period of stimulation.

content was 13 per cent higher (table 2). Determinations on the excited animals were carried out at the same time as the normal series, the rats being taken in pairs of littermates, matched for weight. In 8 of the 10 pairs the phosphocreatine content was higher in the excited animal than in the normal one. The difference from the normal mean in the excited animals was statistically significant when tested by Fisher's *t*-test ($P. < 0.05$).

DISCUSSION

The sequence of changes in the rat brain resulting from stimulation has been studied by analysis after rapid fixation by freezing with liquid air. Animals killed at

and glucose to the brain in emotional excitement, due to adrenaline release, might account for the observed rise in the phosphocreatine level. If the rise in the brain phosphocreatine in emotional excitement is regarded as having physiological significance, it might be considered in the light of Cannon's views as a form of mobilization of the body's reserves as a preparation for action (15).

Considered altogether the present results suggest that the brain ATP level is maintained at the expense of the phosphocreatine and it is therefore relatively stable under all conditions. The phosphocreatine level depends on the balance between the breakdown and resynthesis of high-energy phosphate bonds, each of which processes can be rapid. The phosphocreatine level is determined more by the synthetic capacity of the brain tissue than by the state of functional activity of the brain, so that it may rise in spite of increased demands if the oxygen and glucose supply is increased. The brain ammonia level is also insensitive to small changes in the state of functional activity (6). The brain lactic acid does not rapidly disappear from the tissue when once it has been formed. It can therefore accumulate over an appreciable period of time and can give a useful indication of changes in the state of functional activity of the brain. It is readily formed in anoxia, but the level is also raised by increased functional activity in emotional excitement, when the oxygen supply is good. The brain acetylcholine level falls in emotional excitement and rises in sleep. It responds rapidly to electrical stimulation and to the onset of convulsions. It would thus appear to reflect more closely than any of the other metabolites considered the state of functional activity of the brain. The breakdown of acetylcholine can initiate changes in the phosphate cycle and it may be regarded as a significant factor in determining the various transformations in the phosphate esters which have been considered.

SUMMARY

Electrical stimulation of the rat brain produces *a*) a rapid fall in the phosphocreatine level and *b*) a corresponding transient rise in the hexose phosphate fraction. Over 50 per cent of the total phosphocreatine of the brain is lost after one second of electrical stimulation. The initial changes are followed by *c*) the return of the hexose phosphate fraction to the normal level, accompanied by *d*) the liberation of inorganic phosphate. These changes are evident in brains fixed up to 10 seconds after electrical stimulation and occur before the onset of convulsions. Brains fixed at 15 to 45 seconds after electrical stimulation show *e*) a return of the phosphocreatine and *f*) of the phosphate level to normal. The rate of resynthesis of phosphocreatine under these conditions, as calculated from the slope of the curve, is 1.25 mg. per gm. fresh brain tissue per minute. The adenosine triphosphate fraction shows only a slight fall after stimulation, with a return to normal after about 25 seconds. The brains of rats taken during emotional excitement give a mean phosphocreatine level 13 per cent higher than that of normal controls. There is no significant change in the other phosphate esters.

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SYMPATHOLYTIC EFFECTS OF QUININE AND QUINIDINE¹

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IT HAS been demonstrated in this laboratory that oral doses of cinchona alkaloids cause an increase in renal blood flow in normal dogs (1) and lower the blood pressure of dogs with experimental neurogenic hypertension (2). These results suggest a peripheral vasodilatation and there is considerable support for this hypothesis in the older literature. Nelson (who refers to the earlier literature), reported in 1927 that these drugs antagonize the vasoconstrictor effect of injected epinephrine and 'paralyze' the splanchnic nerves (3, 4).

In these earlier experiments the drugs were given to anesthetized animals by intravenous injection without determination of the concentration of the alkaloids attained in the body fluids. It was the purpose of the investigations reported herein to repeat some of these old experiments, administering the drugs orally as well as by slow intravenous infusion with measurements of plasma alkaloid concentrations and, where possible, under local anesthesia.

METHODS

In the first series of experiments dogs were anesthetized with sodium pentobarbital. They were then prepared for kymographic recording of the blood pressure via carotid artery cannula and mercury manometer. At the same time a slow intravenous infusion of saline was set up and the greater splanchnic nerve exposed in the right thorax, the dogs being ventilated by a mechanical respirator.

The control observations consisted of recording the effect on the arterial pressure of 1) an intravenous dose of epinephrine (0.1-0.6 cc. of 1:10,000 injected into the infusion tubing) and 2) electrical stimulation of the peripheral section of the severed right splanchnic nerve. The electrical stimulation was applied with hand electrodes using 6-volt direct current through an ordinary Harvard-type inductorium arranged to give tetanizing stimuli and with the secondary coil set at 7 cm. The standard duration of this stimulation was 10 seconds.

After recording the control effects, an intravenous infusion of a 200-mg. per cent solution of quinine or quinidine sulfate was started at a rate of 2 to 4 ml/minute. At intervals the effects of epinephrine injection and splanchnic nerve stimulation were tested and blood samples were taken for measurement of alkaloid concentrations².

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² The latter were estimated according to the method of BRODIE, B. AND S. UDENFRIEND. *J. Biol. Chem.* 158: 705, 1945.

In the second series of experiments the effect of orally administered cinchona alkaloids on the circulatory effects of intravenously injected epinephrine was tested on dogs without general anesthesia. The epinephrine was administered in the same manner but blood pressure was recorded photographically from a Hamilton membrane manometer connected to a hypodermic needle inserted, under local anesthesia, into a femoral artery. After control observations the dogs were given repeated 15 mg/kg. doses of one of the alkaloids at 3 to 4-hour intervals, with the experimental recordings being taken at varying times after the last dose.

RESULTS

We were able to confirm the observations of earlier workers that quinine and quinidine antagonize the circulatory effects of injected epinephrine or splanchnic

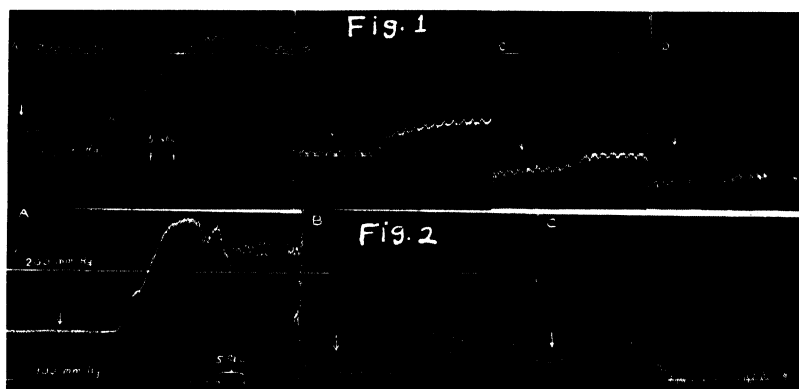


Fig. 1. EFFECT OF QUININE SULFATE on circulatory response to the intravenous injection of 0.4 cc. of 1:10,000 epinephrine. Dog 83, mercury manometer recording from cannulated carotid artery. Arrows mark epinephrine injections. *A* = control. *B* = plasma quinine conc., 2.85 mg/liter. *C* = plasma quinine conc., 4.40 mg/liter. *D* = plasma quinine conc., 7.22 mg/liter. Anesthesia used was sodium pentobarbital.

Fig. 2. EFFECT OF QUINIDINE SULFATE. Dog 82. *A* = control effect. *B* = plasma quinidine conc., 5.34 mg/liter. *C* = plasma quinidine conc., 8.08 mg/liter. Explanation same as for figure 1.

nerve stimulation. The plasma alkaloid concentrations required to achieve a complete block of the pressor effect of the doses of intravenously injected epinephrine as well as the effect of splanchnic nerve stimulation were in the range of 7 to 10 mg/liter but concentrations only half this high markedly reduced the response to both types of stimulation. There was no definite difference correlated with the avenue of administration of the drug nor with sodium pentobarbital anesthesia.

Figure 1 shows sections from the record of an experiment testing the effect of a slow intravenous infusion of quinine sulfate on the circulatory response to the intravenous injection of 0.4 cc. of 1:10,000 epinephrine. Figure 2 shows sections from a similar experiment with quinidine sulfate. The result of a typical experiment showing that quinine and quinidine act in the same manner when administered orally and tested on unanesthetized dogs is shown in figure 3.

The effects of a slow intravenous infusion of quinine on the circulatory response to electrical stimulation of the right greater splanchnic nerve for 10 seconds is shown in figure 4. It can be seen that the pressor effect of this stimulation decreases with

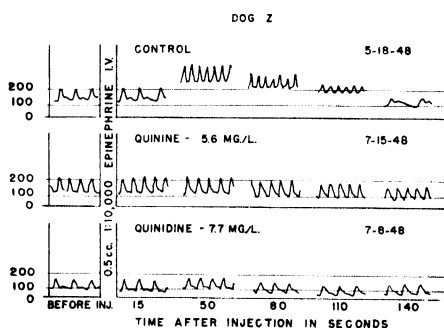


Fig. 3. SECTIONS FROM A CONTINUOUS OPTICAL RECORDING of the arterial pressure of dog Z taken without general anesthesia. The effect of quinine and quinidine, administered orally, on the circulatory response to the intravenous injection of epinephrine is demonstrated.

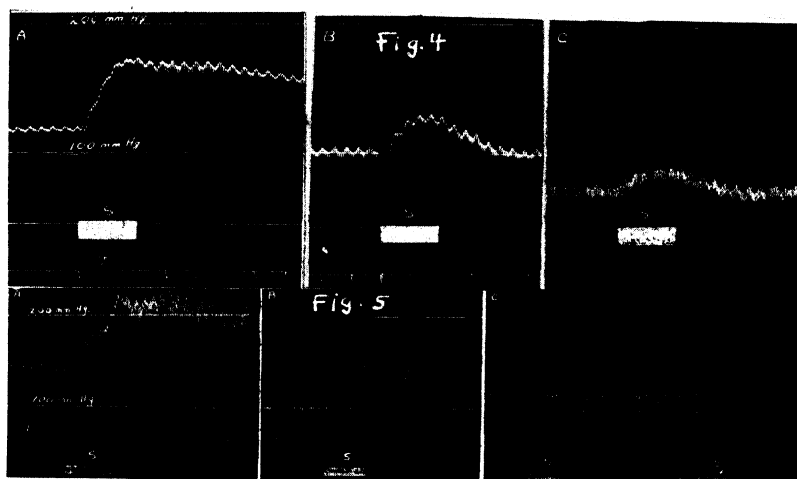


Fig. 4. ACTION OF QUININE on the circulatory effect of stimulation of the right splanchnic nerve Dog 83, mercury manometer recording from cannulated carotid artery. S = 10-second period of Faradization of nerve. T = time in 5-second intervals. A = control effect. B = effect with plasma quinine conc., 4.40 mg/liter. C = with plasma quinine conc., 7.22 mg/liter. Anesthesia used was sodium pentobarbital.

Fig. 5. ACTION OF QUINIDINE. Dog 82. A = control effect. B = effect with plasma quinidine conc., 5.52 mg/liter. C = with plasma quinidine conc., 8.08 mg/liter. Explanation same as for figure 4.

increasing plasma quinine concentration. Figure 5 shows a similar experiment demonstrating the action of quinidine which in this case completely blocked the pressor effect of repeated splanchnic nerve stimulation at a plasma quinidine concentration of 8 mg/liter.

In these experiments quinidine appeared to be somewhat more potent than

quinine and was more likely at higher concentrations to show the phenomenon of 'epinephrine reversal', i.e. a fall in arterial blood pressure after the intravenous injection of epinephrine.

In the experiments with the alkaloids given intravenously there is a progressive fall in blood pressure during the infusion of the drugs which we interpret as being caused by a paralysis of the normal sympathetic tone of the arterioles. However, this effect was not observed to the same degree when the drug was administered orally to the same plasma concentration, a difference which we have noted repeatedly and are at a loss to explain.

DISCUSSION

One of the questions in the minds of those who have observed the blood pressure-lowering effect of the cinchona alkaloids has been whether the effect is achieved by cardiac depression or by peripheral vasodilatation. Nelson (4) concluded that the effect is peripheral "due in part to a depression of the vasomotor endings, and in part to an action directly on the muscle itself." Our observation of renal vasodilatation after these alkaloids are administered supports the theory of peripheral effect (1). In this regard the observations of Ferrer *et al.* (5) on the effects of quinidine on the heart and circulation in man are very interesting. They studied the circulatory effects of single oral doses of quinidine (0.8 gm.) on 18 normal human subjects and 23 patients with cardiovascular disease. In both groups most of the subjects showed a fall in blood pressure but none of them showed a decrease in cardiac output. These authors refer to earlier investigations most of which support the view that quinine and quinidine in ordinary doses do not cause a reduction in cardiac output.

It should be emphasized here that the cinchona alkaloids do not act specifically on sympathetic neuromuscular junctions. It has been repeatedly demonstrated that these agents also block the cardioinhibitory vagus endings (6, 7). The plasma concentration at which this is accomplished is, for quinidine, in the same range as for the sympatholytic effects described above but quinine has a sympatholytic effect at a concentration about half that required for a parasympatholytic effect (7). There is also some evidence for depression of skeletal neuromuscular junctions with rapid rates of stimulation but no measurements of effective plasma concentrations have been made (8). Certainly the effective concentration for paralyzing such endings must be much higher than those required to paralyze the autonomic nerve endings we have investigated for we have seen no evidence of paralysis of respiratory muscles and our unanesthetized animals walk around normally after they have had a sympatholytic dose of quinine or quinidine.

Doses of the same order of magnitude as those given to our dogs are occasionally used in clinical conditions such as malaria, cardiac arrhythmias and myotonia. There is a paucity of information on the plasma concentrations achieved in these therapeutic applications. It is known that the minimum plasma concentration of quinine necessary to suppress a malarial infection in man is from 5 to 10 mg/liter, according to the species and strain of the parasite, with quinidine effective at somewhat lower plasma concentrations (9). Presumably similar concentrations are encountered in other therapeutic uses of these drugs. One wonders whether or not such patients show

symptoms of autonomic blockade. One also wonders whether the sympatholytic effect of these drugs, if it occurs in man as it does in dogs, would be of therapeutic use in such disturbances as hyperfunction of the adrenal medulla (pheochromocytoma) or the neurogenic aspects of hypertension.

It is pertinent to note here that the antagonism between epinephrine and the cinchona alkaloids is a reciprocal one, for Dreisbach and Hanzlik have shown that epinephrine and similar substances counteract the depressor effect of quinine injected rapidly into the blood (10).

SUMMARY

It has been demonstrated that quinine and quinidine will antagonize the pressor effects of epinephrine and of splanchnic nerve stimulation in dogs, thus confirming observations recorded in the earlier literature. This sympatholytic effect occurs with oral as well as intravenous administration of the alkaloids and at plasma concentrations below the range of marked toxicity. The epinephrine antagonism has been demonstrated in unanesthetized dogs. It is felt that the depressor effect of the cinchona alkaloids on the circulation, especially noted on intravenous injection, is caused primarily by peripheral vasodilatation.

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ANTAGONISM OF ADRENOCORTICOTROPIC HORMONE AND ADRENAL CORTICAL EXTRACT TO DESOXYCORTI- COSTERONE: ELECTROLYTES AND ELECTROSHOCK THRESHOLD^{1, 2}

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IT HAS been definitely established that desoxycorticosterone acetate (DCA) induces a retention of sodium (1). On the other hand, reports are at variance regarding the influence of adrenocorticotrophic hormone (ACTH) and of highly oxygenated cortical steroids (the so-called 11,17-oxysteroids which include 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone) on electrolyte balance. ACTH has been reported to induce sodium retention (2-4) and sodium-phoresis (2, 4) in man and to have no consistent influence upon sodium balance in the rat (5). It has been shown that the rate of sodium excretion is increased by 17-hydroxycorticosterone in the rat (6) and dog (7) and by 11-dehydro-17-hydroxycorticosterone in the dog (7). However, 11-dehydro-17-hydroxycorticosterone causes sodium retention in patients with Addison's disease (8). It appears that ACTH and the 11,17-oxysteroids can produce retention as well as excretion of sodium; in contrast, DCA invariably causes sodium retention.

The possibility that at least part of the distortion of electrolyte pattern produced by DCA in the intact animal is due to a relative deficiency of adrenal cortical

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² Presented before the Federation of American Societies for Experimental Biology, Detroit, April 18-22, 1949.

secretion is suggested by the observations that DCA inhibits the discharge of ACTH which normally follows stress (9) and that it induces insulin hypersensitivity (10). In order to test this possibility it was decided to determine the effect of the simultaneous administration of DCA and either ACTH or adrenal cortical extract (ACE) on electrolyte metabolism. Since electroshock threshold has been shown to parallel changes in plasma sodium (11, 12), this index of central nervous system excitability was also measured.

METHODS

Male rats from the Sprague-Dawley farm were used. The animals weighed 200 to 250 gm. at the beginning of the experimental periods.

The technics for determining electroshock seizure threshold and plasma electrolytes in rats have been presented in detail in previous reports (11, 12). At least two days elapsed between successive threshold measurements.

Six 15-mg. pellets of DCA^a were implanted in the subcutaneous tissue of the back of each rat treated with this steroid.

The preparation of ACTH^a employed in Experiment I was exposed to ammonium hydroxide to reduce posterior pituitary activity to approximately 0.1 IU per mg.; the adrenocorticotrophic potency of this preparation was approximately 1/20 that of Armour's ACTH standard (La-1-A) as assayed by the adrenal ascorbic acid-depletion technic of Sayers, Sayers and Woodbury (13). The preparation of ACTH^b used in Experiment III was 50 to 200 per cent as potent as Armour's standard (La-1-A). Posterior pituitary activity was estimated to be less than 0.5 IU per mg. The ACTH preparations were injected subcutaneously in a dose of 2 mg. twice daily.

ACE^c was administered subcutaneously in Experiment II in doses of 1.0 and 0.2 ml. per day.

In Experiment I, 46 rats were divided into 5 groups as follows: *group 1*, 8 non-shocked control rats; *group 2*, 6 shocked control rats; *group 3*, 12 rats implanted with DCA; *group 4*, 10 rats implanted with DCA and injected with ACTH for two periods during the experiment; and *group 5*, 10 rats injected with ACTH for the same two periods during the experiment. The electroshock seizure threshold was followed in *groups 2, 3, 4* and *5* for 40 days after implantation of DCA. The animals were autopsied on the 41st day after DCA implantation, at which time samples of blood were taken for electrolyte analyses.

In Experiment II, 24 rats were divided into three groups of 8 animals each as follows: *group 1*, untreated controls; *group 2*, rats given ACE for three periods during the experiment; and *group 3*, rats implanted with DCA and given ACE for the same three periods. The electroshock seizure threshold was followed in all 24 animals for 72 days. The animals were autopsied 3 hours after the last threshold measurement, at which time blood was withdrawn for electrolyte analyses.

^a Percorten, generously supplied by Dr. Oppenheimer of Ciba Pharmaceutical Company.

^b Preparation 37-L, obtained through the kindness of Dr. Hays of the Armour Laboratories.

^c Kindly supplied by Dr. Brent of Roche-Organon.

^d Lipo-Adrenal Cortex, free of preservative, gift of Dr. Hailman of the Upjohn Company.

In Experiment III, 48 rats were divided into three groups as follows: *group 1*, 16 untreated rats; *group 2*, 16 DCA-implanted rats; and *group 3*, 16 DCA-implanted, bilaterally adrenalectomized rats. The electroshock threshold was followed for 31 days in all animals. At the end of 20 days each of the three groups was divided into two equal *sub-groups a* and *b*; *sub-group b* of each pair received 2 mg. of ACTH twice daily. The rats were autopsied 4 hours after the last threshold measurement; blood was drawn for electrolyte analyses and adrenal weights were recorded.

RESULTS

Excitability of the Central Nervous System

Experiment I. From figure 1 it can be seen that the electroshock seizure threshold of the untreated control rats did not rise above 5 per cent of the initial level at any

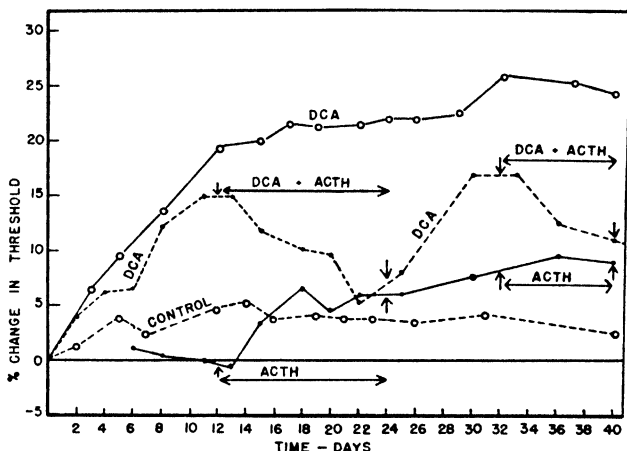


Fig. 1. EFFECT of DCA and ACTH on electroshock threshold. See text for explanation.

time during the experimental period. The slight elevation that did occur has previously been shown to be due to increasing age and weight (14). Implantation of DCA, on the other hand, resulted in a rise in threshold which was initially rapid and then continued, after 16 days, at a slower but nevertheless steady rate. Administration of ACTH to DCA-implanted rats between the 12th and 24th days induced a steady decline in threshold to almost normal levels. After cessation of administration of ACTH the threshold again rose. A course of ACTH from the 32nd to the 40th day produced a second drop in threshold. The supply of ACTH was exhausted at this time and it was not possible to examine whether continued treatment with ACTH would eventually have restored the threshold to normal. ACTH alone induced a slight but insignificant rise in electroshock threshold in animals treated from the 12th to the 24th day of the experiment; in a second course of injections it had no effect whatsoever.

Experiment II. The results of Experiment II are presented in figure 2. The

threshold of the untreated rats rose slightly during the course of the experiment, a finding in keeping with previous observations (14). DCA caused a marked elevation in electroshock threshold. ACE alone produced no significant change in the threshold during any of the periods of injection. However, in DCA-implanted rats, the simultaneous administration of 1.0 ml. of ACE per day restored the excitability of the central nervous system to normal levels within two days. When the injections of cortical extract were discontinued, the threshold increased rapidly and approached a level which would have been attained without ACE treatment. A second course of ACE in smaller dose, 0.2 ml. per day, reduced seizure threshold less rapidly. A third course of 1.0 ml. daily could not be continued sufficiently long to determine the ultimate response to ACE after prolonged DCA administration. However, the diminished response to ACE during the brief final period of its administration suggests that prolonged DCA administration may have made the animals more resistant to the cortical extract.

TABLE 1. PLASMA ELECTROLYTES AND WATER/KG. OF PLASMA FOR RATS OF EXPERIMENT I

TREATMENT		NO. RATS IN POOL	WATER	SODIUM	POTASSIUM	CHLORIDE
			gm.	mEq.	mEq.	mEq.
Untreated	shocked	3	912	144	5.5	107
	unshocked	4	918	141	5.0	107
DCA	shocked	4	918	160	3.5	97
	unshocked	4	922	153	3.8	97
DCA-ACTH	shocked	3	921	147	3.6	100
	unshocked	4	923	146	3.7	100
ACTH	shocked	3	920	148	4.1	105
	unshocked	4	926	147	5.2	106

Experiment III. The results of Experiment III are presented in figure 3. DCA induced a rise in threshold in both intact and adrenalectomized rats. The elevation was greater in the adrenalectomized than in the intact animals. However, the two groups are not strictly comparable because the drinking water of the adrenalectomized, but not that of the intact animals contained 0.9 per cent sodium chloride. ACTH given to DCA-implanted intact rats markedly reduced the threshold; the excitability of the central nervous system was restored to normal by the 10th day after the beginning of ACTH treatment. In contrast to its action in intact animals, ACTH had no effect on the elevated threshold of DCA-treated adrenalectomized animals. It is apparent that ACTH in counteracting the effect of DCA on electroshock threshold, acts via the adrenal.

Electrolytes

Experiment I. In table 1 are presented the results of electrolyte analyses on pooled samples of plasma, taken at the termination of Experiment I. Each of the

various sub-groups of shocked animals was compared with an appropriately treated non-shocked control group. Approximately 24 hours elapsed between the last seizure threshold measurement and the withdrawal of blood for electrolyte analysis.

Repeatedly induced seizures may have had a slight influence upon the concentration of certain electrolytes in plasma. However, the changes were not great enough to mask the more definite alterations produced by hormone treatment. An exception to this general statement will be pointed out below.

No significant change occurred in the concentration of plasma water in any group. DCA, as was to be expected, elevated plasma sodium levels. Simultaneous administration of ACTH and DCA resulted in a level of plasma sodium intermediate between that of untreated and DCA-treated animals; it is to be noted in this connection that the electroshock threshold (fig. 1) of the ACTH-DCA group at the termination of the experiment was intermediate between the thresholds for the untreated and the DCA-treated rats. When ACTH was used alone in Experiment I, it produced

TABLE 2. PLASMA ELECTROLYTES/KG. OF PLASMA FOR RATS OF EXPERIMENT II

TREATMENT	SODIUM	NO. OF RATS	POTASSIUM	NO. OF RATS
	<i>mEq.</i>		<i>mEq.</i>	
Untreated	144.2 \pm 0.6	7	4.58 \pm 0.10	6
ACE	144.3 \pm 0.9 (0.9)	7	4.00 \pm 0.14 (0.9)	8
DCA-ACE	148.1 \pm 0.6 (0.001)	8	3.60 \pm 0.14 (0.001)	8

Figures in parentheses are P values of differences from untreated rats.

a slight elevation in plasma sodium concentration which was accompanied by a slight elevation in seizure threshold (fig. 1).

DCA reduced plasma potassium concentration (table 1), as was to be expected; but ACTH treatment had no influence upon this effect of DCA. ACTH alone caused a reduction in the concentration of plasma potassium in the shocked rats, a finding not confirmed in Experiment III. Plasma chloride concentration was reduced by DCA and partly restored to normal by simultaneous administration of ACTH. ACTH alone had no effect on the level of plasma chloride.

Experiment II. Plasma electrolyte data obtained in Experiment II are presented in table 2. ACE alone did not influence the concentration of plasma sodium or potassium. The group treated with both DCA and ACE had a somewhat elevated plasma sodium concentration; the level was intermediate between the high value for the DCA-treated animals of Experiments I and III and the control values. The electroshock seizure thresholds of the DCA-ACE group were also intermediate between those for DCA-treated and control rats (see fig. 2). Plasma potassium concentration was depressed in the DCA-ACE group to the same low level as the DCA-treated animals of Experiment I.

The electrolyte analyses made at a time when the previously elevated electroshock threshold of the DCA-ACE group was reduced toward normal suggest that the lowering of the threshold of DCA-treated rats by ACE is related to the reduction in plasma sodium concentration. Plasma potassium does not appear to be involved in the changes noted in electroshock seizure threshold. It would be of interest to ascertain the concentrations of plasma electrolytes at a time when ACE has completely restored to normal the elevated threshold of DCA-treated animals.

Experiment III. From table 3 it can be seen that the dose and duration of administration of ACTH to DCA-treated rats were sufficient to restore the elevated plasma sodium levels to normal; this same dosage schedule of ACTH also restored to normal the elevated seizure threshold of the DCA-treated animals (fig. 3). The preparation of ACTH employed in Experiment III, when administered alone, produced a slight but insignificant elevation in plasma sodium concentration; the small elevation in threshold (fig. 3) produced by such treatment is in conformity

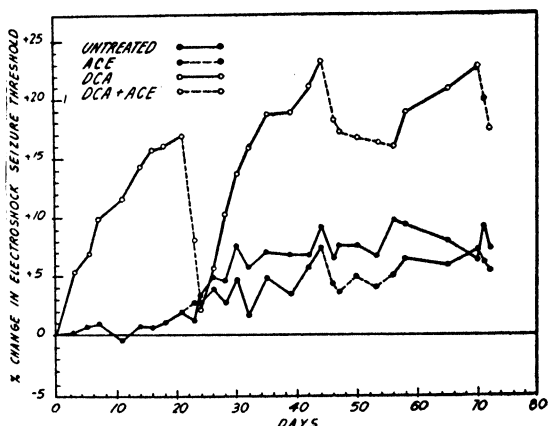


Fig. 2. EFFECT of DCA and ACE on electroshock threshold. See text for explanation.

with the results of Experiment I. In the absence of the adrenals, ACTH had no influence upon the sodium-elevating action of DCA. It appears that ACTH reduces the elevated plasma sodium concentration of the DCA-treated rats by stimulating the secretion of cortical hormones.

From table 3 it is apparent that ACTH produced a slight but insignificant rise in the reduced plasma potassium concentration of the DCA-treated intact rats; ACTH had no influence on the reduced plasma potassium concentration of the DCA-treated adrenalectomized rats.

ACTH only partially restored to normal the lowered plasma chloride concentration in the DCA-treated rats (table 3). DCA lowered plasma magnesium concentration but had no effect upon plasma calcium. ACTH given to DCA-treated rats restored plasma magnesium concentration to normal (table 3).

Adrenal Weights

As can be seen from table 4, the adrenals of DCA-treated rats weighed less than those of untreated rats. This may be interpreted to mean that DCA suppressed

the discharge of ACTH from the pituitary; ACTH produced adrenal hypertrophy in control rats and partially restored adrenal weight to normal in DCA-treated rats.

DISCUSSION

The diversified changes in plasma electrolytes produced by the treatment schedules employed in these studies have presented an opportunity to correlate alterations in excitability of the central nervous system, as measured by electroshock thresholds, with hormone-induced changes in concentration of extracellular electrolytes. Of the various electrolytes studied, sodium has the most constant relation to electroshock threshold; sodium concentration and seizure threshold rise and fall together.

TABLE 3. PLASMA ELECTROLYTES/KG. OF PLASMA FOR RATS OF EXPERIMENT III

TREATMENT	SODIUM	NO. OF RATS	POTASSIUM	NO. OF RATS	CHLORIDE	NO. OF RATS	MAGNESIUM	NO. OF RATS	CALCIUM	NO. OF RATS
	mEq.		mEq.		mEq.		mEq.		mEq.	
Untreated	142.6 \pm 0.8	8	4.70 \pm 0.16	8	105.0 \pm 0.9	6	2.3 \pm 0.04	10	5.50 \pm 0.09	13
DCA	151.2 \pm 1.2 (0.001)	8	3.32 \pm 0.07 (0.001)	8	96.3 \pm 1.0 (0.001)	5	1.78 \pm 0.07 (0.001)	8	5.52 \pm 0.05 (0.6)	8
ACTH	143.9 \pm 0.4 (0.2)	8	4.64 \pm 0.18 (0.8)	8						
DCA-ACTH	143.8 \pm 0.6 (0.2)	8	3.57 \pm 0.12 (0.001)	8	101.3 \pm 1.2 (0.02)	7	2.47 \pm 0.11 (0.1)	8		
Adrenex DCA	148.9 \pm 0.5 (0.001)	7	2.95 \pm 0.09 (0.001)	7						
Adrenex DCA- ACTH	148.5 \pm 0.3 (0.001)	9	2.91 \pm 0.06 (0.001)	9						

Figures in parentheses are *P* values of differences from untreated rats.

Potassium appears to have a minor influence at best, because ACTH administration in DCA-treated rats returns seizure threshold to normal without a corresponding change in potassium concentration. The reduction in plasma chloride concentration which follows DCA-treatment is only partially restored to normal by ACTH at a time when the seizure threshold is normal. An inverse relation exists between the level of magnesium and electroshock threshold. However, evidence available at present makes it impossible to determine whether the plasma magnesium concentration exerts any influence on brain excitability (12). Plasma calcium remained unchanged in the DCA-treated animals, and hence this cation appears to be unrelated to the capacity of DCA to elevate electroshock threshold.

The question arises whether the antagonism of ACE and ACTH to DCA can be explained by the known actions of these compounds on sodium metabolism.

17-hydroxycorticosterone, administered alone, induces sodium retention. The second component of the antagonism manifests itself only in intact animals and is a result of the suppression of adrenal cortical activity by DCA. It has been observed in the present study that DCA produced adrenal atrophy and that the effect of DCA on plasma sodium and electroshock threshold were counteracted by stimulation of the adrenal cortex with ACTH. The concept has been developed that administration of DCA induces a state of steroid hormone imbalance characterized by an excess of DCA (administered steroid) and a deficiency of 11,17-oxysteroids; the deficiency is presumed to arise from inactivity of the adrenal cortex as a result of pituitary inhibition by DCA. Both DCA and 11,17-oxysteroids can inhibit the release of ACTH which normally follows stress (9). Furthermore, Cheng and Sayers (10) have shown that DCA induces a state of insulin hypersensitivity, a finding compatible with the concept that DCA produces a state of relative deficiency of 11,17-oxysteroids. The results of the present study give further support to this concept.

Hypertension, increased size of the heart and kidney, pathological changes which simulate rheumatic lesions of man, 'nephrosclerosis,' periarteritis nodosa, and related cardiovascular changes have been produced in experimental animals by DCA. Selye's interpretation (19) of these effects suggests that human diseases which resemble those of DCA-treated rats can result from adrenal cortical hyperactivity. The findings presented here offer another explanation for the experiments of Selye, namely, that the toxic manifestations of DCA result from a hormonal distortion characterized by an excess of exogenous DCA and a deficiency of 11,17-oxysteroids consequent to inhibition of pituitary adrenocorticotrophic activity. The effect of ACTH and ACE on some of the toxic effects of DCA described by Selye is under investigation at the present time.

SUMMARY

DCA elevated the electroshock seizure threshold in rats. ACTH produced a slight but barely significant rise in seizure threshold, and ACE had no effect on this index of excitability of the central nervous system. The administration of ACTH or ACE restored to normal the elevated electroshock threshold in DCA-treated intact rats; ACTH had no effect on the increased seizure threshold in DCA-treated adrenalectomized rats.

DCA increased plasma sodium concentration and decreased plasma potassium, chloride and magnesium concentrations; DCA had no effect on plasma calcium. ACTH caused a barely significant rise in plasma sodium concentration and had no effect on plasma potassium. ACE had no effect on plasma sodium or potassium concentration. ACTH restored to normal the elevated plasma sodium and the reduced plasma magnesium concentration in DCA-treated rats; it partially restored to normal the low concentration of plasma chloride in DCA-treated rats. However, ACTH did not influence the hypocalcemia induced by DCA. The antagonism of ACTH to DCA in regard to plasma sodium is mediated through the adrenals. ACE reduced the hypernatremia in DCA-treated rats but had no effect on the hypocalcemia.

The results confirm previous observations that the excitability of the central nervous system, as measured by the electroshock seizure threshold, is inversely proportional to the concentration of sodium in the extracellular fluid.

The experiments may be interpreted to mean that the secretion of the adrenal cortex 'normalizes' plasma sodium regardless of the direction of the disturbance in the concentration of this cation, whereas DCA produces sodium retention only.

The antagonistic effects of ACTH and ACE on the DCA-induced elevation of plasma sodium concentration and electroshock seizure threshold provide additional evidence in support of the concept that DCA produces a deficiency of adrenal cortical secretion by inhibiting the adrenocorticotrophic activity of the anterior pituitary.

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INITIAL CHANGES IN THE BLOOD SUGAR OF THE FASTED ANESTHETIZED DOG AFTER ALLOXAN¹

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CERTAIN features of the blood sugar changes in dogs following administration of a diabetogenic dose of alloxan, particularly those relating to alloxan hypoglycemia, have become bases for differences of opinion. The pattern of change has been reported by several groups to be triphasic in nature (1-3), the three phases consisting of initial and final hyperglycemia separated by a phase of hypoglycemia. Shipley and Beyer (4) maintain that the pattern is characteristically tetraphasic in their dogs, the fourth phase being a transient hypoglycemia preceding the other three described above.

Houssay, Orias and Sara (3) have reported the existence of a hypoglycemic phase following administration of 100 mg. of alloxan per kilogram of body weight to dogs under chloralose anesthesia within half an hour after total pancreatectomy. More recently Goldner and Gomori (5) have recorded their failure to observe alloxan hypoglycemia in totally depancreatized dogs under sodium pentothal anesthesia upon administration of diabetogenic doses of alloxan within one half hour after the operation. Several other groups have also published data relating to this problem (6-10).

In the course of a series of investigations performed in this laboratory we have had an opportunity to observe the effects of a number of factors on the blood sugar levels of both normal and recently depancreatized dogs treated with alloxan preparations under standardized conditions. In the normal dogs the effect of alloxan on stainable granules of the beta cells in the Islets of Langerhans has also been studied. We propose to present and discuss these observations, paying particular attention to their possible bearing on the above-mentioned conflicting observations, and to the factors responsible for the production of hypoglycemia following administration of alloxan to the dog.

EXPERIMENTAL

The two alloxan preparations used in our experiments will be referred to as P-Alloxan and K-Alloxan.² By using intravenous sodium amytal as an anesthetic, and following a procedure similar to that described by Houssay, Orias and Sara (3), the patterns of change in blood sugar concentration as functions of time were followed in the 9 groups of dogs listed below: *a*) normal anesthetized; *b*) normal anesthetized, receiving 100 mg/kg. of K-alloxan; *c*) normal anesthetized, receiving 100 mg/kg. of P-alloxan; *d*) anesthetized, freshly depancreatized; *e*) anesthetized, freshly

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² These preparations were obtained from the Pfanstiehl Chemical Company, Waukegan, Ill., and the Eastman Kodak Co., Rochester, N. Y., respectively.

depancreatized, receiving 100 mg/kg. of K-alloxan; f) anesthetized, freshly depancreatized, receiving 100 mg/kg. of P-alloxan; g) treatment as in group b following not less than 6 days on a diet high in carbohydrate and low in fat; h) treatment as in group b following not less than 6 days on a diet high in fat and low in carbohydrate; i) treatment as in group h but including daily injection of protamine-zinc insulin (an average of 7.5 units/dog/day) for not less than 6 days, replaced by crystalline insulin on the second day before experiment and with no insulin thereafter. Groups of dogs receiving treatment similar to those in groups a, b and e were also studied, using either chloralose or morphine plus ether as anesthetic.

The dogs receiving the high-fat low-carbohydrate diet consumed a daily average of 5.2 gm. of raw beef suet and 17.5 gm. of stewed meat per kilogram of body weight. To this daily diet 0.1 gm. of choline chloride was added. Those receiving the diet low in fat and high in carbohydrate consumed a commensurate amount of minced cooked meat plus 10.4 gm. of sucrose per kilogram of body weight mixed with it. All animals received a daily supplement of one teaspoonful of dry brewer's yeast. 'Unconditioned' animals had been fed the regular laboratory ration for dogs, consisting of a mixture of cooked meat, vegetables and cooked cereal grains. This ration is estimated to be intermediate between the above two diets in carbohydrate and fat content. Diets were fed once each day, with water supplied *ad libitum*.

The experiments were all started in the early morning, the animals having been fasted overnight. Adult female dogs of intermediate weight were used throughout. Fasting blood sugars were determined in duplicate during an 8- or 9-hour period, by the use of the micromethod of Miller and Van Slyke (11). In groups b, c, e, f, g, h and i isotonic neutral saline containing one per cent by weight of one of the alloxan preparations was passed continuously from a burette into a tibial vein until a dose of 100 mg. of alloxan preparation per kilogram of body weight had been delivered. The average time required for this infusion was 6 ± 2 minutes. The dry alloxan preparation was added to the saline just prior to administration. In groups e and f an average time interval of 0.44 ± 0.07 hours elapsed between the mid-point of the pancreatectomy operation and the start of alloxan infusion.

Determinations of insulin content were made on the freshly removed pancreases of some of the dogs of groups b, e, f, g, h and i. In all but groups e and f the pancreases were removed for insulin extraction at 9 hours after alloxan administration. Extractions were made according to the method of Scott and Fisher (12), and the insulin potency of such extracts was measured to within a standard error of ± 10 per cent by a mouse convulsion method. Sections of pancreas from some of the normal and 9-hour dogs were stained by Bowie's method³ (13) to permit observation of beta-cell granulation.

Data on blood sugar concentrations for individual dogs of all groups except g, h and i are shown in table 1. Apart from the 58 dogs, on which blood sugar measurements are presented in table 1, similar observations were made on 4 other dogs. These were excluded on the basis of post-mortem findings of post-operative abdominal hemorrhage in 3 cases and of death at 6 hours after alloxan with pulmonary edema and hemoconcentration in the fourth. In addition to the 22 dogs of groups g,

³ Modification by D. W. Wilson and W. S. Hartroft (to be published).

TABLE I. RESPONSE OF FASTING BLOOD SUGAR FOLLOWING ADMINISTRATION OF 100 MG/KG. OF ALLOXAN TO UNCONDITIONED ADULT FEMALE DOGS ANESTHETIZED WITH SODIUM AMYTAL¹

GROUP AND TREATMENT	INITIAL BODY WT.	INITIAL	TIME IN HOURS										R ²
			0.0	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	
kg.			Fasting blood sugar in mg. %										mg. %/hr.
Na-Amytal Anesthesia													
Normal:	6.2	86	82	83	85	87	84	80	93	97	90	80	
Controls	11.4	82	85	87	87	87	86	81	81	81	83	80	85
	7.7	83	86	89	90	80	84	88	79	85	84	85	89
Average.....	8.4	84	84	86	87	85	85	85	84	88	86	82	
S.E.	±1.5	±1	±1	±2	±1	±2	±1	±3	±4	±5	±2	±1	
Normal:	9.8	93	75	114	103	177	160	131	45	31	29	(29)	84
K-Alloxan	6.6	75	87	105	113	182	181	176	154	120	104	83	75
	9.1	81	90	59	117	177	189	173	152	62	62	27	24
	9.6	80	108	68	169	222	223	167	77	44	53	32	61
	4.8	93	94	36	42	45	85	183	226	205	107	53	59
Average.....	8.0	84	91	76	143	161	168	166	131	92	71	45	
S.E.	±1.0	±4	±5	±15	±29	±30	±23	±9	±32	±30	±18	±11	
Normal:	6.1	83	94	57	112	168	166	143	70	36	30	47	61
P-Alloxan	7.7	84	84	102	(119)	(139)	137	61	50	48	47	49	63
	5.9	84	102	46	(115)	(154)	122	91	34	30	34	29	41
Average.....	6.6	84	93	68	115	154	142	98	51	38	37	42	
S.E.	±0.6	±1	±5	±17	±2	±6	±13	±24	±26	±5	±5	±6	
Freshly-depancreatized	10.8	80	79	85	83	95	90	104	130	146	164	171	
Controls	6.3	70	68	73	71	114	137	178	206	209	229	231	
	6.8	96	118	125	130	123	148	184	197	203	184	188	
Average.....	8.0	82	88	94	95	111	125	155	178	186	192	197	
S.E.	±1.5	±8	±15	±16	±18	±8	±18	±27	±24	±20	±19	±18	
Freshly-depancreatized	10.9	72	88	97	102	113	160	182	230	229	238	244	258
K-Alloxan	6.4	74	110	115	160	261	232	262	289	361	327	267	248
	8.5	86	87	118	133	104	157	159	164	193	210	245	265
	10.7	91	96	200	191	169	147	127	120	110	104	104	102
	5.5	79	158	220	252	233	202	163	173	153	152	142	150
	9.6	88	105	128	135	175	189	169	159	123	124	84	(78)
	6.7	79	91	142	195	243	181	157	75	58	50	40	
	4.8	83	91	137	151	173	169	119	113	105	95	98	
	5.2	69	58	63	59	50	48	54	53	59	63	66	
Average.....	7.6	80	98	136	153	169	165	155	153	155	151	143	
S.E.	±0.8	±3	±3	±16	±19	±23	±17	±19	±25	±31	±30	±20	
Freshly-depancreatized	9.1	73	74	71	82	75	86	74	49	48	67	48	27
P-Alloxan	5.2	97	131	133	119	89	42	127	127	149	154	178	
	9.1	91	98	168	169	170	134	82	66	44	38	29	33
	6.4	83	134	160	153	130	155	186	167	125	112	121	(140)
	9.8	80	114	168	183	138	87	73	68	55	56	55	68
	10.9	82	106	113	117	157	130	132	140	172	188	209	25
	5.6	89	114	127	122	118	117	113	76	89	106	124	
	12.6	99	118	208	202	182	154	138	120	111	111	104	89
	4.7	78	74	123	106	100	135	169	204	223	219	221	
Average.....	8.2	86	101	141	139	129	116	122	113	113	117	121	
S.E.	±0.9	±3	±7	±13	±13	±12	±12	±13	±17	±20	±20	±23	

TABLE 1.—Continued

GROUP AND TREATMENT	INITIAL BODY WT.	INITIAL	TIME IN HOURS											R ¹
			0.0	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	
kg.			Fasting blood sugar in mg. %											mg. %/hr.
Chloralose Anesthesia														
Normal: Controls	6.7	84	86	86	86	69	77	74	80	86	86	92	107	
	9.4	101	106	112	105	97	88	68	72	74	86	85	88	
	7.9	91	77	82	89	90	80	117	122	144	82	85	82	
	10.0	79	93	78	74	116	103	87	73	70	85	79	77	
Average.....	8.5	89	91	89	86	93	89	87	87	94	85	85	89	
S.E.....	±0.8	±5	±6	±8	±6	±10	±5	±11	±11	±17	±1	±3	±7	
Normal: K-Alloxan	7.0	84	99	112	150	159	132	102	71	64	40	26	34	31
	7.2	91	106	112	171	189	103	105	131	54	42	51	74	
	9.3	97	92	128	177	217	196	123	75	55	60	70	41	
	10.2	81	78	114	154	178	172	146	90	50	62	57	47	
Average.....	8.4	88	94	126	166	179	143	111	90	50	48	53		
S.E.....	±0.8	±4	±6	±9	±5	±18	±24	±14	±15	±4	±8	±8		
Freshly-depancreatized K-Alloxan	8.0	93	129	158	169	174	152	144	138	102	78	73	49	19
	6.7	94	177	190	192	187	186	200	222	237	238	264	237	
	7.1	89	105	111	83	99	71	45	36	58	56	56	61	28
	8.2	91	63	85	84	109	139	162	185	224	237	235	212	
	10.7	77	109	148	212	182	158	154	143	125	91	106	107	
Average.....	8.2	89	119	139	148	151	142	141	145	150	140	148	134	
S.E.....	±0.7	±4	±19	±18	±27	±19	±19	±26	±30	±35	±40	±43	±39	
Morphine and Ether Anesthesia														
Normal: Controls	6.7	89	170	157	148	121	104	112	101	82	79	80	80	
	7.6	93	169	149	129	116	101	106	102	90	96	91	85	
	5.7	102	218	192	165	143	123	115	105	90	82	75	78	
	8.2	81	90	100	89	72	71	69	72	82	79	81	83	
Average.....	7.1	91	162	150	133	113	100	101	95	86	84	82	82	
S.E.....	±0.6	±4	±25	±19	±16	±15	±11	±11	±8	±2	±4	±3	±2	
Normal: K-Alloxan	5.2	84	150	384	370	281	189	148	100	74	72	82	87	28
	5.5	86	100	274	280	227	187	148	95	69	62	48	45	32
	6.8	92	108	195	221	203	183	167	118	101	87	77	72	13
	7.5	83	92	140	208	220	196	210	181	110	81	59	43	28
Average.....	6.3	86	113	248	270	234	189	169	124	89	76	67	62	
S.E.....	±0.6	±2	±13	±53	±37	±17	±3	±15	±20	±10	±6	±8	±11	
Freshly-depancreatized K-Alloxan	6.6	86	284	328	351	425	426	423	431	422	421	413	436	
	7.0	82	195	232	238	242	247	266	262	279	286	307	303	
	10.9	91	221	204	203	207	208	250	194	204	218	226	234	
	7.0	80	221	233	221	197	183	195	214	214	207	212	214	
	8.6	90	237	240	246	243	240	174	156	182	182	199		
Average.....	8.0	86	232	248	252	263	261	262	252	260	263	271		
S.E.....	±0.8	±2	±15	±21	±26	±42	±43	±44	±68	±45	±43	±40		

¹ Values in parentheses are those for which blood sample was not obtained directly on the hour indicated. Such values were obtained by linear interpolation using off-the-hour measurements. ² R represents rate of blood sugar fall in mg. %/hour, interpolated at 90 mg. % as the intermediate phase of hypoglycemia is entered.

h and *i* for which blood sugar data are presented in table 2, 5 others underwent the pre-experimental period of diet, or of diet plus insulin. Of these one died during administration of the anesthetic, and the other 4 died before the end of the 9-hour period of observation, 2 with pulmonary edema. Results of estimations of pancreatic insulin for all groups are summarized in table 3.

OBSERVATIONS

Characteristics of the Blood Sugar Response to Alloxan in Unconditioned Dogs.

The fasting blood sugar of the normal anesthetized dog not previously subjected to treatment with diet or insulin (i.e. unconditioned) is observed to pass through a more or less characteristic sequence of changes following administration of alloxan (table 1). These changes and their frequency of appearance are listed below in the order in which they occur:

1) An occasional brief fall in blood sugar occurring within the first hour after alloxan (initial hypoglycemia). Such a fall was observed in 2 of 4 of the normal dogs of this series which were treated with alloxan under chloralose anesthesia, in 5 of 8 of those alloxanized under sodium amytal anesthesia, and in 0 of 4 of those receiving alloxan while anesthetized with morphine plus ether.

2) A rise in blood sugar above that of the corresponding normal control group (intermediate hyperglycemia). Only one exception to this rise was observed in the series of experiments on unconditioned dogs presented here.⁴

3) A fall in fasting blood sugar to values below the control group level (intermediate hypoglycemia). This occurred within 9 hours after alloxan in all but one animal. In this dog, anesthetized with sodium amytal, the blood sugar had fallen to the initial level at 9 hours and appeared to be still falling at that time.

Two types of change were observed in the fasting blood sugar of anesthetized freshly depancreatized dogs receiving 100 mg/kg. of K- or P-alloxan. In 18 of all such dogs studied the blood sugar rose to and remained at hyperglycemic levels throughout the period of observation. The remaining 10 developed intermediate phases of hypoglycemia. In these animals the sequence and timing of blood sugar changes were comparable with those observed in the anesthetized normal alloxanized dog, except that the initial fall in blood sugar concentration was absent. The proportion of recently depancreatized dogs in which the fasting blood sugar fell below the initial normal level following administration of alloxan shows an inverse association with the magnitude of the rise in the blood sugar level during the intermediate phase of hyperglycemia (table 4).

b) Influence of Diet and Administered Insulin on Characteristics of the Blood Sugar Response to Alloxan. Groups of adult female dogs receiving the treatment with diet and insulin described earlier, prior to intravenous administration of 100 mg/kg. of K-alloxan under sodium amytal anesthesia, showed certain characteristic responses which have been summarized in table 5. It is noted that both the frequency

⁴ One other exception has been observed among 13 unconditioned normal dogs treated with intravenous doses of alloxan ranging from 100 to 200 mg/kg. without anesthesia. While the blood sugar did pass through a maximum (following an initial fall) at 2 hours after alloxan administration, the maximum value was below that of the initial normal value for this animal. Of these 13 dogs 4 showed initial hypoglycemia at 0.5 hours after alloxan.

of production and amplitude of the initial phase of hypoglycemia increase as the conditioning diet becomes lower in carbohydrate content and higher in fat, and that initial hypoglycemia is most pronounced in those dogs which were pre-treated with insulin while on the high-fat diet.

TABLE 2. RESPONSE OF FASTING BLOOD SUGAR FOLLOWING ADMINISTRATION OF 100 MG/KG. OF K ALLOXAN TO CONDITIONED ADULT FEMALE DOGS ANESTHETIZED WITH SODIUM AMYTAL

TREATMENT CONDITIONING	BODY WT.	INI- TIAL	AFTER AN- ESTH.	TIME IN HOURS										R ¹
				0.0	0.5	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	
	kg.			Fasting blood sugar in mg. %										mg. %/hr.
High carbohy- drate diet	10.0	90	78	92	54	148	147	138	112	83	48	27	29	30
	8.6	65	66	62	66	103	211	235	200	181	124	73	42	47
	5.7	75	85	74	48	120	183	186	158	120	65	51	51	52
	6.8	75	72	83	44	65	152	148	102	56	39	20	23	46
	8.0	75	78	76	125	(200)	186	152	137	77	54	44	30	45
	8.3	71	81	75	100	200	266	255	228	133	54	34	27	75
	13.8	80	103	95	72	145	223	209	179	157	132	77	51	45
Average.....	8.7	76	80	80	73	140	196	189	159	115	74	47	36	49
S.E.....	±1.0	±3	±4	±4	±11	±24	±16	±17	±25	±18	±14	±8	±4	
High fat diet	7.9	73	80	79	37	52	32	39	61	59	40	30	19	
	6.3	69	68	69	47	82	145	154	125	44	27	26	19	76
	6.4	73	78	72	(53)	66	56	79	123	141	114	79	37	36
	7.7	83	83	83	63	66	124	148	104	44	34	31	27	55
	9.6	71	83	82	64	74	66	83	78	33	26	25	25	
	9.5	76	80	77	69	144	187	211	191	161	116	35	32	73
	9.0	87	82	111	88	83	88	81	(84)	81	70	65	59	
	13.8	81	84	82	73	110	178	217	195	128	56	(43)	35	69
	14.2	84	81	77	78	164	166	182	164	108	42	31	32	64
Average.....	9.4	77	80	81	64	93	116	133	125	89	58	41	32	62
S.E.....	±1.0	±2	±2	±4	±5	±13	±19	±21	±16	±16	±12	±6	±4	
High fat diet + insulin	9.6	84	80	91	40	33	35	31	37	39	34	26	42	
	7.1	77	87	93	57	133	150	147	101	64	49	43	36	39
	5.7	74	80	80	32	76	98	151	218	192	181	164	87	69
	5.0	86	88	82	49	68	72	66	79	53	31	27	18	
	10.0	79	77	81	48	52	105	132	126	101	68	48	38	31
	13.7	73	73	76	88	147	211	216	202	147	85	(45)	(42)	51
Average.....	8.5	79	81	84	52	85	112	124	127	99	75	59	44	48
S.E.....	±1.3	±2	±2	±3	±8	±19	±25	±27	±29	±25	±23	±21	±9	

¹ R represents the rate of blood sugar fall in mg%/hour interpolated at 90 mg% as the intermediate phase of hypoglycemia is entered.

The reverse is found to hold for the intermediate phase of hyperglycemia. In addition to the greater average rise in blood sugar in the carbohydrate-fed dogs than in the fat-fed ones during this phase, the maximum was reached more quickly in the former group.

The appearance of an intermediate phase of hypoglycemia was the most consistent characteristic response of dogs to alloxan. It was observed in all but 2 of the 27 normal dogs completing the 9-hour period of observation after administration of K-alloxan under sodium amytal anesthesia and in all other normal dogs injected with 100 mg/kg. of alloxan under chloralose or ether anesthesia. In the 2 exceptions the courses of the blood sugar curves indicate that hypoglycemia would have been observed shortly after the 9th hour, had the experiments not been terminated at that time. The group-average times after alloxan administration at which this phase began show no characteristic differences attributable to diet.

c) *Influence of Brand of Alloxan Used.* The average courses of blood sugar change in the normal anesthetized dog treated with either K- or P-alloxan are biometrically indistinguishable during the first 2 hours after alloxan administration. However, the fall from the hyperglycemic maximum to hypoglycemia occurs approximately 2 hours earlier in dogs receiving P-alloxan than in those treated with K-alloxan, and the differences in average blood sugar in these 2 groups are sufficiently great at the 4th, 5th, 6th and 7th hours after alloxan administration to appear statistically significant (table 1).

In groups of freshly depancreatized dogs receiving either P- or K-alloxan under sodium amytal anesthesia, the average blood sugar values for the group treated with P-alloxan once again fall below the corresponding values for the group which received K-alloxan, the difference first appearing 2 hours after alloxan administration and showing some degree of significance at the 2nd, 3rd and 4th hours.

Samples of these 2 alloxan preparations have been submitted to physical and chemical analyses in this laboratory. While K-alloxan proved to be readily soluble in water, 10.1 per cent of P-alloxan was found to consist of relatively insoluble matter. A summary of the findings based on chemical analyses of the soluble and insoluble fractions is presented in table 6. This observed difference in chemical composition provides a basis for explaining what appear to be statistically significant differences in the blood sugar responses of normal and of freshly depancreatized dogs to these 2 preparations.

With these suggestive differences in response in mind, it is worthy of note that in a survey of 70 randomly selected publications dealing with the diabetogenic and associated effects of alloxan, the source of the alloxan used was mentioned in only 13 cases, and tests of identity or purity were indicated in only 4. Since alloxan preparations are obtainable from at least 7 different commercial sources, and in some cases have been prepared locally, it would be in the interests of all who are working in this field if some statement of the purity or source of the alloxan used were provided in publication.

d) *Influence of Anesthetics.* Observations were made on groups of dogs anesthetized with one of the following substances: 1) chloralose (100 mg/kg., i.v., 0.8 per cent in isotonic saline), 2) sodium amytal (50 mg/kg., i.v., 5 per cent in isotonic saline), 3) morphine (0.25 grains subcutaneously) followed by ether given by mask prior to and during pancreatectomy and administration of alloxan, or for an equivalent time to control animals in amounts sufficient to maintain anesthesia.

No significant difference between corresponding groups in average blood sugar

response was noted at any time after administration of chloralose or sodium amytal to the following groups of animals: 1) normal control dogs; 2) normal dogs receiving K-alloxan; 3) freshly depancreatized dogs receiving K-alloxan (table 1).

On the other hand, when morphine plus ether was used as anesthetic, the blood sugar was immediately elevated to a significant degree above the corresponding values for animals under chloralose or sodium amytal. Following administration of K-alloxan to either normal or to freshly depancreatized dogs anesthetized with morphine plus ether, the blood sugar rose within the first hour to approximately the same very high level in each case. In the freshly depancreatized animals it remained at or above this level for the remainder of the period of observation. In the normal group receiving K-alloxan, the blood sugar fell continuously after reaching this high value, and passed below that of the control group by the 6th hour after alloxan.

e) *Influence of Alloxan on Beta Cell Granulation and Insulin Content of Pancreas.* The insulin content of dog pancreas removed under sodium amytal anesthesia 9 hours

TABLE 3. GROUP AVERAGE VALUES, OBTAINED BY EXTRACTION AND ASSAY, FOR INSULIN CONTENT OF PANCREAS IN NORMAL FASTED ADULT DOGS, AND IN SIMILAR ANIMALS 9 HOURS AFTER INTRAVENOUS INJECTION OF 100 MG. OF K-ALLOXAN/KG. OF BODY WEIGHT

GROUP	NO. OF DOGS	INSULIN	INSULIN	INSULIN
		units/gm. of pancreas ± S.E.	units/kg. body wgt. ± S.E.	units/dog ± S.E.
Unconditioned normal.....	23	1.91 ± 0.18	4.34 ± 0.31	36.0 ± 6.0
Unconditioned 9-hr. alloxan.....	9	1.97 ± 0.21	4.18 ± 0.41	30.0 ± 4.8
High CHO diet 9-hr. alloxan.....	4	1.91 ± 0.36	4.58 ± 0.75	43.5 ± 9.1
High fat diet 9-hr. alloxan..	9	2.23 ± 0.33	4.97 ± 0.71	45.2 ± 6.9
High fat diet plus insulin 9-hr. alloxan.....	4	1.49 ± 0.18	3.89 ± 0.73	32.2 ± 5.0

after administration of alloxan, is compared in table 3 with that of unconditioned normal dogs similarly anesthetized but not given alloxan. Sections of tissue from the central part of the pancreas were taken from many of these animals and placed in Bouin's fixative, and later stained by a modification of Bowie's method for visualization of the beta-cell granules.

The following conclusions are drawn from the data in table 3: 1) the average amount of insulin per gram of pancreas in normal dogs receiving the regular laboratory maintenance rations does not differ significantly from that of similar unconditioned animals depancreatized 9 hours after the intravenous injection of 100 mg/kg. of K-alloxan.

2) The average amount of insulin per gram of pancreas in normal dogs following 6 or more days of conditioning on a high carbohydrate or a high fat diet does not differ significantly from the corresponding figures for those groups described in 1). The insulin per gram of pancreas in dogs which received exogenous insulin while placed on the high fat diet is lower than that of all other groups.

3) If insulin per kilogram of body weight or insulin per dog is considered as an index of insulin content of pancreas, no significant differences are found to exist between any 2 of the groups.

4) In those alloxanized dogs for which both insulin per gram of pancreas and index of beta-cell granulation were determined, the former by assay, the latter by independent histological estimation, a significant degree of positive linear correlation is found to exist. Although degenerative changes of the nuclei were obvious in the beta cells of those animals pancreatectomized 9 hours after alloxan, the granules in the cytoplasm were easily visualized, and no change from normal in their numbers could be discovered on inspection of sections stained by the modification of Bowie's method (fig. 1a, 1b).

These findings are in contrast to the beta-cell degranulation reported by Goldner and Gomori (14), which began at about the same time as the intermediate phase of hypoglycemia after injection of dogs with alloxan.

f) *Associations Between Features of the Blood Sugar Response to Alloxan and Insulin Content of Pancreas.* The magnitude M of the initial fall in the fasting blood sugar between 0.0 and 0.5 hours after the administration of 100 mg/kg. of K-*alloxan* to adult female dogs anesthetized with sodium amytal, and the rate R of blood sugar fall in these animals, as the intermediate phase of hypoglycemia is entered, have been compared with various indices of the insulin content of pancreas (table 7). It is found that M showed significant degrees of linear correlation with insulin per gram of pancreas as well as with the histological index of beta-cell granulation, but not with pancreatic insulin per kilogram of body weight. No significant degree of linear correlation between M and R , or between R and any of the indices of insulin content of pancreas was identified.

DISCUSSION

General anesthesia appeared highly desirable in our experiments, since total pancreatectomy was performed in some groups of animals. Furthermore we were anxious to make certain of our experiments comparable with those of Houssay, Orias and Sara, who used chloralose as an anesthetic. However, in view of the experience or suggestions of others (4, 5, 15), the possibility that the use of anesthetics may have modified our results must be considered.

The phases of blood sugar change in the alloxanized normal dog anesthetized with chloralose or sodium amytal were found equivalent in all points, and to correspond in sequence and time of appearance with those recognized as occurring in the normal unanesthetized alloxanized dog (unpublished observations on 8 dogs). It is therefore concluded that neither of these anesthetics modified the fasting blood sugar level of normal dogs to a degree comparable with the changes induced by the alloxan itself.

The occurrence of an intermediate phase of hypoglycemia in some of the freshly depancreatized alloxanized dogs, while they were anesthetized with chloralose or sodium amytal, indicates that this hypoglycemia was not simply an artefact caused by one or other of these agents. This conclusion is supported directly for sodium amytal by the absence of such a hypoglycemic phase in 3 freshly depancreatized

dogs anesthetized with this agent but not receiving alloxan (table 1). Thus our observations do not provide support for the suggestion advanced by Shipley and Beyer (4) and by Griffiths (10), that the hypoglycemia observed by Houssay *et al.* in freshly depancreatized dogs treated with alloxan, may have been dependent upon the prolonged use of chloralose anesthesia.

The present observations do not provide support for the suggestions of Shipley and Beyer (4) and Lukens (15) that barbiturate and possibly chloralose anesthesia render animals more sensitive to the toxic effects of alloxan. In only one of our experimental groups did more than one animal die with either barbiturate or chloralose anesthesia. The exception was that group in which the animals were pretreated with insulin, and hypoglycemia following alloxan was early and profound (mortality 33 per cent within 9 hours, table 5). Reference to the original data of Houssay, Orias and Sara (3) shows no hypoglycemia and no mortality in 1- and 2-day depancreatized

TABLE 4. PROPORTION OF RECENTLY DEPANCREATIZED DOGS SHOWING A FALL IN FASTING BLOOD SUGAR BELOW THE INITIAL NORMAL VALUE FOLLOWING INTRAVENOUS INJECTION OF 100 MG/KG. OF ALLOXAN

ANESTHETIC	ALLOXAN PREPARATION	FASTING BLOOD SUGAR (F.B.S.) BEFORE AND AFTER ALLOXAN (mg. %)			FREQUENCY OF FALLS IN F.B.S. AFTER ALLOXAN BELOW INITIAL VALUE	
		Initial F.B.S. \pm S.E.	Group Av. F.B.S. at 1.0 hr. \pm S.E.	Difference \pm S.E.		
Morphine plus ether....	K-	86 \pm 2	252 \pm 26	166 \pm 26	fraction	%
Sodium amytal.....	K-	80 \pm 3	153 \pm 19	73 \pm 20	0/5	0
Chloralose.....	K-	89 \pm 4	148 \pm 27	59 \pm 28	3/9	33
Sodium amytal.....	P-	86 \pm 3	139 \pm 13	53 \pm 14	2/5	40
Chloralose ¹	Not known	91 \pm 6	102 \pm 14	11 \pm 16	5/9	56
					9/9	100

¹ Since the treatment of dogs in the above series of experiments was patterned on that used by Houssay, Orias and Sara, their results with chloralose are shown in table 4 for purposes of comparison.

dogs within 8 hours after alloxan, while the mortality was 33 per cent within 6 hours in similarly treated recently depancreatized dogs, all of which developed hypoglycemia. Chloralose anesthesia was administered to all of these animals. It therefore appears that the toxic effects in such animals are not observed unless hypoglycemia is coexistent, and in this circumstance even unanesthetized dogs show a high mortality after alloxan (8). With the aid of suitable diet and insulin, we have maintained several of the dogs for weeks or months after administering 100 mg/kg. of K-alloxan following pancreatectomy under sodium amytal anesthesia.

Lukens (15) has suggested that ether be used if anesthesia is needed at the time alloxan is given. In experiments designed specifically to study the early effects of alloxan on the blood sugar, advantage cannot be taken of this suggestion, since, as is well known, ether anesthesia in the dog is accompanied by an extensive rise in the blood sugar level (table 1), caused in large part by mobilization of glucose from the liver (16). However, the action of ether is transient and our dogs were conscious when the intermediate phase of alloxan hypoglycemia developed.

The data of tables 1 and 2 confirm the existence of an initial phase of hypoglycemia following intravenous administration of a diabetogenic dose of alloxan to the dog. Shipley and Beyer (4) have surmised that this phase is caused by endogenous insulin, and Lukens (15) has suggested that, in view of the occasional mitoses and beta-cell degranulation observed in rabbits 24 hours after small doses of alloxan (17), the initial hypoglycemic phase may be caused by a stimulating action of alloxan on the islands of Langerhans. It is of interest to recall in this connection the significant degrees of negative linear correlation which were found to exist between the depth of fall of blood sugar between 0.0 and 0.5 hours after alloxan, and insulin content of pancreas (measured histologically as intensity of beta-cell granulation, and assayed as units of insulin per gram of pancreas). If such relations were determined merely by the magnitude of the pancreatic depot of insulin, one would expect a very large initial hypoglycemic phase in the freshly depancreatized dog receiving alloxan. However, no such phase has been observed in any of our freshly depancreatized animals after administration of K-*alloxan*⁶ under any of the anesthetics employed.

The fact that a preliminary period of fat-feeding, and particularly one of fat-feeding plus administered insulin, enhances the initial hypoglycemia, also fits no ready explanation apart from the stimulation hypothesis. In terms of such an hypothesis, it would appear that the 'rested' insulin-producing cells of such dogs were capable of greater response to stimulation by alloxan than the 'active' ones of the carbohydrate-fed animals. If beta cells are initially stimulated by alloxan to release insulin, the duration of the response to stimulation must be short, since alloxan is transformed into other substances within minutes of its intravenous injection (18), and since necrotic changes are detectable in the beta cells shortly thereafter (19, 20).

In view of experimental evidence that the intermediate phase of alloxan hyperglycemia is caused by mobilization of carbohydrate from the liver (3), the more pronounced hyperglycemia which occurred in the carbohydrate-fed dogs than in fat-fed ones was to be expected. The observation of an even less prominent intermediate hyperglycemia in the fat-fed dogs receiving exogenous insulin agrees with the finding that insulin administered to normal dogs causes a decrease in the amount of hepatic glycogen below that of control animals not receiving insulin (21). The differences in the rapidity with which the blood sugar becomes elevated in this phase emphasize the need for caution in interpreting the associations existing between the magnitude *M* of the initial fall in blood sugar after alloxan and insulin factors noted in the preceding paragraph. It is obvious that *M* will be determined not only by the effectiveness of factors which depress the blood sugar level, but also by those which initiate the intermediate phase of hyperglycemia.

As indicated in the preceding discussion of the effects of anesthetics, our data lend support to the observation of Houssay, Orias and Sara (3) that an intermediate phase of hypoglycemia can occur in the freshly depancreatized dog after the administration of alloxan. However, in our experiments this phenomenon was observed in only 10 out of 28 such animals while the above-mentioned authors originally reported its occurrence in all of the 9 dogs used in their published experimental results. When,

⁶ One such animal showed a very minor fall (3 mg.%) between 0.0 and 0.5 hours after administration of P-*alloxan*.

however, their series was completed, only a minority of their dogs exhibited this hypoglycemia (Houssay, personal communication). Our results indicate that the frequency of appearance of the intermediate phase of hypoglycemia is inversely associated with the degree to which the blood sugar level is elevated in the preceding phase of hyperglycemia. In the light of this inverse association, it would be predicted that a large proportion of the recently depancreatized dogs described by Houssay *et al.* (3) would show alloxan hypoglycemia since in their animals the preceding hyperglycemia was slight (table 4).

A phase of hypoglycemia occurring several hours after the administration of alloxan to a fasting animal could only be caused by one or more of the following changes: *a*) Loss of glucose from the body by a phloridzin-like action of alloxan; *b*) increase in rate of withdrawal of glucose from the blood by other tissues of the body, an insulin-like or insulin-mediated action; *c*) reduction in the normal rate of

TABLE 5. SUMMARY OF RESPONSE CHARACTERISTICS IN CONDITIONED GROUPS OF NORMAL ADULT FEMALE DOGS AFTER RECEIVING THE STANDARDIZED TREATMENT WITH K-ALLOXAN UNDER SODIUM AMYTAL ANESTHESIA. PERCENTAGES SHOWN ARE TAKEN RELATIVE TO THE NUMBER OF DOGS SURVIVING TO 9 HOURS AFTER ALLOXAN

PRE-ALLOXAN CONDITIONING TREATMENT	NO. OF DOGS		BLOOD SUGAR FALL AT 0.5 HOURS AFTER ALLOXAN		INTERMEDIATE PHASE OF HYPERGLYCEMIA			INTERMEDIATE PHASE OF HYPOGLYCEMIA	
	Start	End	Occur- rences	Amplitude of Fall \pm S.E.	Occur- rences	Group Av. Max. Blood Sugar \pm S.E.	Group Av. Tim. of Max.	Occur- rences	Group Av. Time ¹ of Start
			No. %		No. %	mg. %	hr.	No. %	hr.
CHO diet.....	8	7	4 57	6 \pm 32	7 100	196 \pm 16	2.3	7 100	5.6
Regular diet.....	5	5	4 80	14 \pm 18	5 100	168 \pm 23	2.7	4 80	6.1
Fat diet.....	10	9	8 89	16 \pm 11	6 67	133 \pm 21	3.5	9 100	5.0
Fat diet plus insulin.	9	6	5 84	32 \pm 8	4 67	127 \pm 30	3.8	5 83	5.4

¹ Taken as the time at which the fasting blood sugar fell through 90 mg%. Hence dogs whose fasting blood sugar did not rise above this level are not included in this figure.

supply of glucose to the blood from those tissues which perform this function in the fasting dog.

Of these, possibility *a*) can be ruled out immediately for our dogs, since at most only traces of glucose have been found to be excreted prior to the phase of permanent hyperglycemia following the alloxan administration. This was established by placing such alloxan-treated dogs in metabolism cages and testing the fluid excreta collected during the first 9 hours after alloxan for the presence of reducing substances. Since alloxan administered to unanesthetized normal dogs occasionally produced vomiting such excreta were included in some analyses.

Direct evidence upon which to form an opinion concerning possibility *b*) for the depancreatized animal is at present limited to a single experiment performed by Corkill, Fantl and Nelson (22). These authors used a spinal eviscerated cat into whose circulating blood glucose was infused at a fixed rate. The blood sugar concentration of this preparation was observed to remain at an effectively constant value for over 4 hours, although 50 mg. of alloxan per kilogram of body weight was

injected halfway through this observation period. While confirmatory evidence on this aspect of the problem is desirable, this result supports the conclusion that an increased rate in uptake of glucose by the tissues is not a factor in the production of hypoglycemia in the freshly depancreatized dog. It is therefore indicated, by the process of exclusion, that the hypoglycemia observed in such animals results from a reduction in the rate of supply of glucose to the blood caused by administration of alloxan. The finding by Houssay and Gerschman (7) that a dilute solution of alloxan perfused through frog liver suppresses spontaneous glycogenolysis in such a manner that it cannot be revoked by the action of adrenaline suggests the site and also a possible mechanism by which alloxan produces hypoglycemia in the recently depancreatized dog.

Our finding of an association between the amplitude of the initial hyperglycemic phase at 1.0 hour after alloxan⁶ and the frequency of occurrence of hypoglycemia in the recently depancreatized alloxanized dog (table 4) supports the view that the magnitude of the depot of labile glycogen in this organ following alloxan administration may be a factor in determining the course of the blood sugar in such animals. This view is in agreement with the observed inability to produce an intermediate phase of alloxan hypoglycemia in the dog 48 hours after pancreatectomy (3), since within this time the liver becomes essentially free of glycogen, while within half an hour after pancreatectomy under chloralose or amytal anesthesia most of the liver glycogen is still present (21). It is, however, difficult to suggest a mechanism by which labile liver glycogen favors the production of hypoglycemia in a depancreatized dog.

Concerning the absence of hypoglycemia in the experiment of Goldner and Gomori with dogs receiving alloxan half an hour after pancreatectomy, we note that the fasting blood sugars of 3 of the 4 dogs used had risen between pancreatectomy and alloxan administration to levels between 45 and 63 per cent above the initial normal value. While a single feature such as this should be considered together with other relevant factors such as source, dose, site and rate of injection of alloxan, and the nature of the diet consumed by animals in the pre-experiment period, it should receive consideration, in the light of our findings, when the occurrence and cause of alloxan hypoglycemia are being debated.

The effect of the passage into the blood stream of active endogenous insulin cannot be disregarded in considering the causes of the intermediate phase of hypoglycemia in normal animals treated with alloxan. The average time interval which elapsed following alloxan administration before the normal dogs of table 1 entered the intermediate phase of hypoglycemia was 5.7 ± 0.4 hours.⁷ One might therefore expect that, if insulin leached from the necrosed beta cells of the Islets of Langerhans were a factor in its production, the onset of the hypoglycemic phase would be pre-

⁶ By taking the blood sugar level at 1.0 hours after alloxan as an index, the effects of both operative procedures and administration of alloxan on the blood sugar are included. A relation similar to that indicated above is obtained if the average rise in blood sugar at the maximum of the first hyperglycemic phase is compared with the frequency of occurrence of a subsequent hypoglycemia.

⁷ The corresponding figure for the freshly depancreatized dogs of table 1 which developed hypoglycemia is 4.1 ± 0.8 hours.

ceded or accompanied by a fall in the insulin content of pancreas. Contrary to this expectation no fall has been observed to occur within the first 9 hours after alloxan administration (table 3) and, at an earlier date, this observation was cited as evidence that endogenous insulin did not play a part in the production of the initial part of the alloxan hypoglycemia (23).

However, even in the absence of a significant fall in insulin content of pancreas, pancreatic insulin could still contribute to the initial part of the intermediate phase of hypoglycemia if alloxan acted either to increase the rate of formation and release of insulin in the pancreas, or to increase the effectiveness of endogenous insulin. By comparing the beta cells of figure 1*b* with the normal ones of figure 1*a*, the advanced stage of necrosis of these cells at 9 hours after alloxan is obvious, although the insulin content of pancreas and beta cell granulation density still remain within normal limits. Were it possible for such necrosed beta cells to maintain the increased rate of insulin turnover prior to this time, suggested by Banerjee (6), the available experi-

TABLE 6. ANALYSIS OF CHEMICAL COMPOSITION OF K-ALLOXAN AND P-ALLOXAN, USING THE TITRIMETRIC METHOD OF ARCHIBALD (1945)

FRACTION	CHEMICAL COMPOSITION	K-ALLOXAN	P-ALLOXAN
		%	%
Insoluble	Alloxantin		4.7
	Alloxan-H ₂ O		2.7
	Unidentified		2.7
Soluble	Alloxan-H ₂ O	100.0	81.7
	Unidentified		7.8
Lost in separating fractions			0.4
Total		100.0	100.0

mental evidence indicates that the insulin content of pancreas would almost certainly be altered toward a new equilibrium level (24). Our data do not indicate that such a change has taken place. Houssay, Orias and Sara (3) have followed changes in the blood sugar level after administration of alloxan to the 24-hour depancreatized dog with the pancreas of a normal dog grafted into its carotid artery, but likewise have found no evidence of hypersecretion of insulin.

It has been shown that all of the sulfur in molecules of active insulin is in the form of disulfide linkages (25), and that even a partial reduction of the disulfide linkage to sulfhydryl groups renders the insulin physiologically inactive (26). In view of its effectiveness in oxidizing sulfhydryl groups, alloxan might be expected not only to leave the physiological activity of active insulin unchanged (27), but also it might even cause a reactivation of some insulin inactivated by sulfhydryl-bearing substances. Reports of increased sensitivity to exogenous insulin associated with lowered levels of glutathione and cysteine in the tissues (28, 29), coupled with the rapid and extensive falls in the blood concentrations of these substances following

intravenous administration of alloxan (18), indicate an additional way in which the effectiveness of insulin might become increased in the alloxan-treated animal.

It should be emphasized that lack of observation of a significant fall in insulin content of pancreas during the first 9 hours after alloxan does not rule out the possibility that a slow or late fall, slight enough to be commensurate with the standard error of measurement and hence undetected by the method of analysis employed, may have occurred. In such a case if factors which normally limit the effective life of insulin *in vivo* are reduced by alloxan, the combination of effects could conceivably make it possible for a relatively small fraction of the pancreatic insulin to influence the intermediate hypoglycemic phase at its onset.

Reference should be made at this point to the consistent appearance of an intermediate phase of hypoglycemia in normal dogs receiving the standard treatment with alloxan while anesthetized with ether. The large initial rise in blood sugar level in these animals is seen to be commensurate with that which occurred in similarly anesthetized dogs receiving alloxan after pancreatectomy (table 1). If the intermediate phase of alloxan hypoglycemia were produced only by extrapancreatic factors, the blood sugar responses of these 2 groups to alloxan should have been similar. Since they differ consistently in this regard one is led to believe that endogenous insulin must have played a part, directly or indirectly, in the onset of hypoglycemia in the dogs with intact pancreas.

The average rate R of blood sugar fall at the start of hypoglycemia in these animals was 25 ± 4 mg. per cent per hour as compared with 48 ± 9 and 56 ± 12 mg. per cent per hour in similarly treated animals anesthetized with chloralose and sodium amytal, respectively. The difference between the former and either of the latter values is significant, and could reasonably be considered to measure the effectiveness of the hypoglycemic mechanism found to operate in the freshly depancreatized dog treated with alloxan providing the liver has not been made to release sugar in excess.

If the above line of reasoning is valid, then R for the recently depancreatized dog developing hypoglycemia after alloxan should also be less than that observed in the normal. This appears to be so, for those animals of table 1 for which R could be estimated, the average value being 32 ± 3 mg. per cent. The apparent dependence of R on more than one mechanism for the production of hypoglycemia could be a reason why no significant degrees of association of this factor with indices of the insulin content of pancreas were resolved out in table 7.

The published data indicate that the insulin content of pancreas has fallen to very low levels by 24 hours after alloxan in the dog (30, 31). Houssay, Orias and Sara (3) have also shown that at this time the release of insulin by such a pancreas, when grafted into a depancreatized dog, was far below normal in 5 out of 6 of the cases tested. If then all of the insulin passing into the blood stream between the 9th and 24th hours after alloxan consisted simply of that present in the pancreatic depot at the time when the alloxan was administered, the average rate of insulin release during this time would be approximately 0.2 units/hr/kg. of body weight. Soskin and Allweiss (32) found that intravenous administration of insulin to depancreatized dogs at the rate of 0.07 and 0.08 units/hr/kg. required the simultaneous

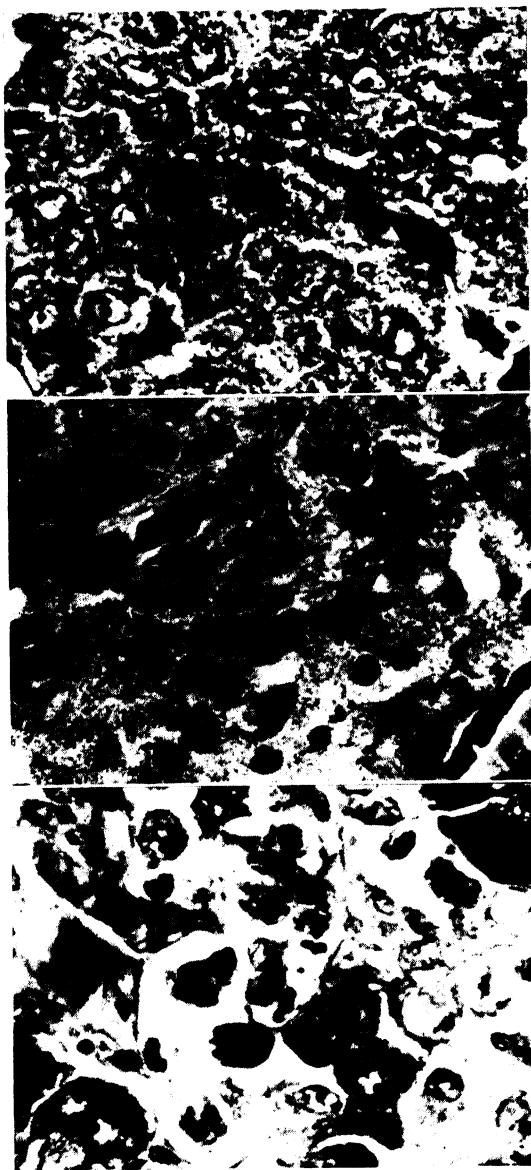


Fig. 1. Effect on beta cell granulation and insulin content of pancreas of 100 mg/kg. of K-alloxan administered to normal adult female dogs. Bouin's fixative. Modified Bowie's stain. Magnification $\times 875$. A. (upper). Normal control: 2.35 units of insulin/gm. of pancreas. Fasting blood sugar: 85 mg.%. B. (center). Typical, 9 hours after alloxan: 2.21 units of insulin/gm. of pancreas. Terminal fasting blood sugar: 30 mg.%. C. (lower). Typical, 24 hours after alloxan: 0.36 units of insulin/gm. of pancreas. Terminal fasting blood sugar: 127 mg.%.

administration of 0.25 gm. dextrose/hr/kg. to maintain a normal blood sugar level. Hence, in surveying the factors which produce an intermediate phase of hypoglycemia following administration of alloxan to the normal dog, it is clearly to be expected that the release of pre-formed insulin at more than twice this rate in the fasting alloxanized dog would more than suffice to account for hypoglycemia occurring later than the 9th hour after alloxan.

In view of the above observations and the presence of an intermediate phase of alloxan hypoglycemia in the freshly depancreatized dog, it becomes increasingly apparent that alloxan probably produces hypoglycemia in the normal dog by complex means. However, methods which have been used to date to study the causes of alloxan hypoglycemia in the normal animal, including our own, provide only circum-

TABLE 7. DEGREE OF LINEAR CORRELATION FOUND TO EXIST BETWEEN SPECIFIC CHARACTERISTICS OF THE BLOOD SUGAR PATTERN OF CHANGE IN NORMAL ADULT FEMALE DOGS RECEIVING 100 MG/KG. OF K-ALLOXAN UNDER SODIUM AMYTAL ANESTHESIA, AND PANCREATIC INSULIN FACTORS.

Factors correlated are described in the list beneath the table. The probability of obtaining by pure chance the degree of linear correlation shown is designated as p .

FACTORS CORRELATED	COEFFICIENT OF LINEAR CORRELATION $r \pm S.E.$	NO. OF DOGS USED	p
1 and 2	-0.15 ± 0.23	18	over 0.5
1 and 3	-0.062 ± 0.27	14	" 0.5
1 and 4	-0.11 ± 0.27	14	" 0.5
1 and 5	-0.062 ± 0.27	14	" 0.5
1 and 6	-0.14 ± 0.31	11	" 0.5
2 and 3	$+0.053 \pm 0.27$	14	" 0.5
2 and 4	-0.47 ± 0.10	16	0.07
2 and 6	-0.61 ± 0.10	11	0.04
4 and 6	$+0.62 \pm 0.16$	15	0.01

Factors used in above correlations, by number: 1. Rate of fall of fasting blood sugar at 90 mg. % as the intermediate phase of hypoglycemia is entered. 2. Magnitude of the fall in fasting blood sugar between 0.0 and 0.5 hours after alloxan administration. 3. Pancreatic insulin per kilogram of body weight, obtained by assay. 4. Insulin per gram of pancreas, obtained by assay. 5. Insulin per dog, obtained by assay. 6. Index of beta-cell granulation, obtained without foreknowledge of factor 4, by histological methods.

stantial evidence on which to base conclusions concerning the contribution of endogenous insulin. Much of this indirect evidence can be interpreted in more than one way. Thus the demonstration of a diabetic type of glucose tolerance, and the presence of a dip in blood sugar in place of an intermediate phase of hypoglycemia in the fasted phloridzinized rabbit treated with alloxan has been interpreted by Banerjee and Bhattacharya (9) as evidence that alloxan hypoglycemia is pancreatic in origin. They assume that the pancreatic depot of insulin in these animals has been reduced by the phloridzin treatment. Reference to the experiments of Soskin and his associates on factors governing glucose tolerance in the dog (33), and knowledge that the liver glycogen content of phloridzinized-fasted animals is abnormally low (34) provide a second rational basis on which the findings of Banerjee and Bhattacharya could be interpreted without implicating an excessive release of insulin as

the hypoglycemic factor. It appears probable that a direct answer to the contribution made by endogenous insulin to the production of alloxan hypoglycemia in the normal animal will be obtained only when it is possible to measure accurately the insulin content of blood.

SUMMARY AND CONCLUSIONS

The effects of anesthetic, diet and insulin on the initial blood sugar changes in normal adult female dogs following administration of alloxan preparations have been studied. Lack of uniformity in the properties and effects of different alloxan preparations has been described. The existence of an initial phase of hypoglycemia after alloxan administration in the normal dog has been confirmed and the relationship of this effect to diet and insulin has been investigated. The existence of an intermediate phase of hypoglycemia appearing under certain circumstances in the recently depancreatized dog has been confirmed.

Reasons are presented which suggest that the corresponding phase of alloxan hypoglycemia in the normal dog is of more complex origin than that observed in the depancreatized animal. Indirect evidence suggests that in the former the action of insulin may be involved but this point is not established. A significant degree of positive linear correlation has been observed between the insulin per gram of pancreas, estimated by bio-assay in the pancreas of alloxan-treated dogs and an histological index of beta-cell granulation.

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CHANGES IN THE BLOOD OF THE RAT FOLLOWING EVISCERATION

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THIS laboratory group is engaged in a study of factors which affect the survival and metabolic behavior of the eviscerate rat. The data of this report represent some of the hematologic and biochemical changes from normal in the blood of the rat 48 hours following evisceration.

METHODS

Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. The two-stage procedure of evisceration has been described (1). When the animals reached a weight of 250 ± 2 grams, they were anesthetized (intraperitoneal injection of 18 mg. of cyclopentenyl-allyl-barbituric acid sodium) and were subjected to the second stage of evisceration. Asepsis was preserved in both stages of the operation. Intravenous injections of solutions containing 0.9 per cent sodium chloride and a glucose load of 44 mg. per 100 grams of rat per hour with regular insulin (Lilly) in the amount of 4 units per 24 hours per rat were made by a continuous injection machine which delivered fluid from each of 6 syringes at the rate of 20 cc. in 24 hours. The infusions were made into the saphenous vein of the right hind leg and were started within 5 minutes following removal of the liver. The animals were enclosed in a cabinet with temperature constant at $26^{\circ} \pm 0.5^{\circ}\text{C}$. At the end of 48 hours the blood was drained from a needle inserted into the abdominal aorta. No anti-coagulant was required.

The following procedures were used: Prothrombin time in whole blood, the macro method of Ziffren, Owen, Hoffman and Smith (2); hemoglobin was determined by use of the Haden-Hausser hemoglobinometer; cell volume, Van Allen (3). All nitrogen determinations were by the micro-Kjeldahl procedure. Non-protein nitrogen, Robinson, Price and Hogden (4); the value for non-protein nitrogen was subtracted from total nitrogen to obtain the value for total plasma proteins. Fibrin was precipitated according to Johnston and Gibson (5) and the nitrogen content of the supernatant fluid was subtracted from the nitrogen value of the total proteins to obtain the fibrin value. Globulin was precipitated by the method of Kingsley (6) and the nitrogen content of the supernatant fluid was determined. The nitrogen values for non-protein nitrogen and fibrin were subtracted from this value to give the nitrogen value of albumin. The sum of the values for fibrin and albumin was subtracted from the value for total proteins to give the value for globulin. Plasma amino acids, Hamilton and Van Slyke (7) as modified by Schott, Rockland and Dunn (8); serum calcium, Clark and Collip (9); serum inorganic phosphorus, Müller (10); plasma sodium and potassium, the flame photometer according to Overman and Davis (11); CO_2 combining power of plasma, Peters and Van Slyke (12); plasma chloride, Whitehorn (13); plasma bilirubin, Malloy and Evelyn (14).

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RESULTS

Blood Clotting. After 48 hours of evisceration the blood of the rat does not clot. This has been true of all of the hundreds of rats in this laboratory that have survived 48 hours and longer following evisceration. Thromboplastin was added to blood samples from 12 rats 48 hours following evisceration. No clots formed in any of the samples. It is assumed that the failure of the blood to clot is partially due to depletion of the prothrombin level of the blood.

Blood Cells (Table 1). There was no significant change in the concentration of red cells or of hemoglobin but there was a marked reduction in the number of white cells following evisceration. The percentage of polymorphonuclear cells among the total number of white cells was increased from a normal value of 24 per cent to 54 per cent in the eviscerate rats. Since the total white count of the eviscerate series was small the actual number of polymorphonuclear cells was also reduced but to a much smaller extent than the lymphocytes. The percentage of lymphocytes in the total white count was decreased from a normal average of 75 to 47 per cent in the

TABLE 1. HEMATOLOGIC CHANGES IN RAT BLOOD 48 HOURS FOLLOWING EVISCERATION. AVERAGES DIFFERENCES AND THEIR STANDARD DEVIATIONS

	CONTROL		EVISCERATE		DIFFERENCE
	No. of Rats	Average	No. of Rats	Average	
Hematocrit (% cells).....	12	42.8 \pm 0.65	18	39.9 \pm 1.63	2.9 \pm 1.75
Hemoglobin (gm.% whole blood).....	12	12.7 \pm 0.2	28	12.8 \pm 0.4	0.1 \pm 0.46
Red blood cells (million/cu.mm.).....	12	7.24 \pm 0.23	28	7.40 \pm 0.34	0.16 \pm 0.41
White blood cells (cu.mm.)....	12	11678 \pm 1013	28	2797 \pm 305	8881 \pm 1058
Prothrombin time (sec.).....	12	20	12	infinite	

eviscerate series. No bilobed white cells were noted in the control series whereas 2 per cent of the white cells of the eviscerate rats were bilobed. Nucleated red cells occurred rarely in the control series but an average of 6 nucleated red cells were noted per 100 white cells in the eviscerate series.

Plasma Proteins (Table 2). There was a significant reduction in the total plasma proteins of the eviscerate rats. There was a loss of globulin and fibrin but the greatest part of the loss was albumin. In the control series the A/G ratio was 0.96 and in the eviscerate series the A/G ratio was 0.58.

Plasma Amino Acids and Non-Protein Nitrogen (Table 2). There was a significant increase in the non-protein nitrogen of the plasma following evisceration which was due in part to an increased concentration of amino acids. The other non-protein nitrogen constituents of plasma have not been measured after 48 hours of evisceration. The concentration of urea in the plasma of 24 rats was determined 24 hours after evisceration. The average value of 19.6 mg. per cent was not significantly different from the average value of 20.4 mg. per cent for 31 control rats. When it is considered that the eviscerate rat given continuous intravenous infusions of 20 cc.

per 24 hours is in positive water balance and that these animals excreted an average of 18 mg. each of urea nitrogen during this period the possibility is indicated that extra-hepatic synthesis of urea may have occurred.

Serum Calcium and Inorganic Phosphorus (Table 2). There was a significant decrease in serum calcium 48 hours after evisceration but serum inorganic phosphorus was not significantly altered.

Plasma Sodium and Potassium (Table 2). No significant change in plasma sodium was noted following evisceration but a definite rise occurred in plasma potassium. The plasma and ascitic fluid from the 48-hour eviscerate rats were usually but not always pink in color suggesting that some hemolysis of cells had occurred which would release cellular potassium into the plasma.

Plasma Chloride and CO₂ Combining Power (Table 2). A definite increase in plasma chlorides occurred in the eviscerate rats which is assumed to be related to the development of acidosis which was evidenced by the low value for CO₂ combining power.

Plasma Bilirubin (Table 2). Although no measurable amounts of bilirubin occur in the plasma of normal rats the eviscerate animals become severely jaundiced. Doctor C. J. Watson, University of Minnesota School of Medicine, studied the plasma from one 48-hour eviscerate rat of this series. The total bilirubin content was 3.7 mg. per cent of which 1.2 mg. was the prompt reacting type.

Gross Pathology. As the eviscerate rat approaches death there is an accumulation of fluid in the abdominal and thoracic cavities. In some instances the thoracic cavity fills with fluid to the extent that respiration becomes impossible. We have not measured the volumes of fluid which accumulate in the body cavities but the amounts are large. In a number of instances the abdominal as well as the thoracic cavity has been almost filled with fluid. The fluid is generally pink, indicating the presence of hemoglobin, but clear, straw colored fluid has been observed at the time of death in some instances. In the 48-hour eviscerate rat the ascitic fluid contains significant amounts of albumin and globulin but thorough quantitative studies have not been made.

At necropsy the eviscerate rat shows multiple hemorrhages which have occurred spontaneously throughout the body. They occur most frequently in the kidneys, ureters and bladder but have been noted in almost every other part of the body including the lungs and brain.

During the first 24 hours following evisceration the kidneys secrete urine but during the second 24 hours anuria develops and the kidneys appear congested and edematous.

DISCUSSION

The results of this study provide some clues as to the causes of death of the eviscerate rat and some basis for planning improved intravenous feeding to prolong survival. The escape of fluid from the vascular system may occur to such an extent that respiration fails for mechanical reasons. However, if the fluid is drained from the abdominal and thoracic cavities the animal may survive for a few hours longer but will then die for unidentified reasons.

The decrease in white cells, especially of lymphocytes, may be related to the increased susceptibility of the eviscerate rat to infections, an important factor in limiting survival (15). The development of acidosis probably does not favor survival and the possibility that the administration of alkaline substances would have a favorable effect upon survival should be investigated.

The depletion of prothrombin and the resulting failure of the blood to clot is probably the basis for the diffuse hemorrhages which occur throughout the body of the eviscerate rat. We do not know the relationship of these vascular changes to the escape of water into the body cavities and the depletion of the plasma proteins but they probably have a causal relationship. Theoretically the decrease in plasma proteins favors the escape of water from the blood. The factors responsible for the

TABLE 2. CHEMICAL CHANGES IN RAT BLOOD 48 HOURS FOLLOWING EVISCERATION. AVERAGES, DIFFERENCES AND THEIR STANDARD DEVIATIONS

	CONTROL		EVISCERATE		DIFFERENCE
	No. of Rats	Average	No. of Rats	Average	
Total protein (gm.% plasma)...	26	5.83 \pm 0.045	23	3.24 \pm 0.062	2.59 \pm 0.076
Albumin (gm.% plasma).....	24	2.59 \pm 0.069	23	1.08 \pm 0.042	1.51 \pm 0.081
Globulin (gm.% plasma).....	21	2.70 \pm 0.074	23	1.85 \pm 0.044	0.85 \pm 0.086
Fibrin (gm.% plasma).....	23	0.58 \pm 0.043	23	0.34 \pm 0.039	0.24 \pm 0.058
N.P.N. (mg.% plasma).....	27	36.0 \pm 0.79	23	59.9 \pm 1.01	23.9 \pm 1.28
Amino acids (mg.% plasma)...	27	4.94 \pm 0.060	33	12.0 \pm 0.305	7.06 \pm 0.311
Calcium (mg.% serum).....	31	11.6 \pm 0.12	33	9.3 \pm 0.11	2.3 \pm 0.16
Phosphorus (mg.% serum)....	31	8.9 \pm 0.08	28	9.0 \pm 0.28	0.1 \pm 0.29
Sodium (mg.% plasma).....	30	339.0 \pm 3.46	30	336.0 \pm 1.82	3.0 \pm 3.91
Potassium (mg.% plasma).....	30	17.5 \pm 0.46	30	22.5 \pm 0.48	5.0 \pm 0.67
Chloride (mg.% plasma).....	34	378.0 \pm 2.75	22	429.0 \pm 6.20	51.0 \pm 6.78
CO ₂ (vol.% plasma).....	34	64.3 \pm 0.79	26	31.4 \pm 1.18	32.9 \pm 1.42
Bilirubin (mg.% plasma).....	30	no measurable amount	35	4.2	4.2

decrease in plasma proteins, other than leakage into the ascitic fluid, are not known. In addition to a decrease in formation, due to the absence of the liver, the plasma proteins may be utilized for tissue synthesis or for energy purposes (16). The extent to which nitrogenous compounds are exchanged between the plasma and tissues in the eviscerate rat is not known.

The concentration of red cells and of hemoglobin remained at normal values in the eviscerate rat. This should not be regarded as satisfactory evidence that the volume of the blood remained normal. In the absence of measurements of blood volume and of blood flow and judging from gross observations it is our impression that the active volume of blood is gradually reduced due to spontaneous hemorrhages and to the stagnation of blood in the peripheral tissues. The heart rate decreases from an initial rate of over 300 per minute to an average of about 65 beats per minute in the 48-hour eviscerate rat.

The procedure of evisceration is one of the most drastic insults that a mammalian organism can withstand for even a few days and it cannot be safely said that its metabolic processes are normal except for the absence of the intra-abdominal organs. However, if a metabolic process can be demonstrated in the absence of one or several organs it is conclusive proof that the remaining tissues are capable of carrying out that process and when a metabolic adjustment can be demonstrated in the eviscerate animal it is reasonable to assume that the peripheral tissues possessed these same capacities before the liver and other organs were removed.

In addition to our interest in the metabolic behavior of the eviscerate rat we would like to determine those factors which influence its survival. Prolongation of survival might be accomplished by two lines of approach which we have not yet fully explored: 1) Improved intravenous feeding intended to compensate for the abnormal changes found to occur in the eviscerate animal. For example, the administration of substitutes for the plasma proteins, an optimal balance of vitamins and inorganic compounds and the correction of acidosis might prolong survivals. 2) The administration of substances occurring in liver such as prothrombin and hypothetical hormone-like substances which might be present in liver extracts would test the extent to which the survival of the eviscerate rat could be extended by substitution therapy.

SUMMARY

Male rats of 250 grams weight were eviscerated and given continuous injections of glucose (44/100/hr.) and insulin for 48 hours. At this time the blood failed to clot even after the addition of thromboplastin. There was no significant change in the concentration of hemoglobin or of the red cells, but the white cell count was decreased to about 24 per cent of normal with the greatest extent of the loss being in the number of lymphocytes. There was a significant fall in all of the plasma proteins with albumin showing the greatest extent of decrease. The increase in plasma non-protein nitrogen was due in part at least to a rise in amino acids. The serum inorganic phosphorus was not significantly changed but there was a decrease in serum calcium. The plasma potassium was increased, possibly due to hemolysis of red cells, and there was no change in plasma sodium. All of the eviscerate rats developed acidosis with an accompanying rise in plasma chlorides. Although there was no measurable amount of bilirubin in the plasma of normal rats the eviscerate animals became severely jaundiced by 48 hours.

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INFLUENCE OF VARIATION IN ENVIRONMENTAL TEMPERATURE AND THYROID STATUS ON GROWTH AND FEED CONSUMPTION OF THE MALE MOUSE¹

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A STUDY of the literature (1, 2) shows that mild hyperthyroidism accelerates growth in young animals of several species. In severe hyperthyroidism, where the basal metabolism is considerably elevated, growth retardation occurs.

The decrease in thyroid secretion rate of rats (3) and mice (4) that occurs in animals exposed to high environmental temperatures is usually accompanied by a decreased food intake and growth rate. This is, presumably, one of the homeothermic mechanisms that enables the animal to maintain a normal body temperature when exposed to heat stress. Little information is available, however, on the response to thyroidal stimulation in animals kept at high temperatures. The present study was undertaken, therefore, to determine the effect on the growth and food intake of modifications in both the environmental temperature and the thyroid status. As shown by the studies of Robertson (5) and Koger and Turner (1) the laboratory mouse is particularly responsive to the growth-stimulating effect of thyroidal substances. It was thought that this species would also be highly sensitive to modifications in environmental temperature because of its high metabolic rate and surface area relative to its body mass.

MATERIALS AND METHODS

Young male mice weighing 12 to 14 gm. were obtained from the Rockland Farms, New City, New York. Upon arrival the mice were kept in an air-conditioned laboratory at 24°C. and 45 to 55 per cent relative humidity for an acclimatization period of one week. Electric lights were turned on from 8 A.M. to 5 P.M. daily. The mice were then numbered individually, and apportioned to the various experimental groups such that the average body weights were similar. In each experiment, one series of mice was kept in the animal room at 24°C. and a second series was placed in glass-topped incubators maintained at 30° ± 0.5°C. Finely ground Purina 'Laboratory Chow' and water were given *ad libitum*. Special feeding trays designed to prevent wastage were used so that the food intake of each group could be accurately

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² Government of Pakistan Scholar.

determined. Water consumption was estimated by weighing daily the inverted type watering bottles before and after replenishing the water supply. The requisite amounts of thyroprotein (Protamone)³ and thiouracil⁴ were weighed to an accuracy of 0.1 mg. and thoroughly mixed with the weighed quantity of feed in a mechanical mixer. The mice were removed from the incubators for a short period once each week in order to weigh them. Feed and water consumption and notes on the general

TABLE I. GENERAL INFORMATION ABOUT THE EXPERIMENTS

GROUP NO.	DOSAGE	DURATION OF EXPER.	NO. OF MICE	AVERAGE WT.		MEAN WEIGHT GAIN IN GM. WITH S.E.
				At Start	At End	
		wk.		gm.	gm.	
<i>Exper. I. Maintained at 24°C.</i>						
1	Control	4	10	18.0	27.9	9.9 ± .188
2	.2% TH ¹	4	10	17.5	25.9	8.4 ± .191
3	.025% TP ²	4	5	17.3	28.7	11.4 ± .387
4	.05% TP	4	10	17.4	29.8	12.4 ± .224
5	.1% TP	4	9	18.0	28.1	10.1 ± .253
6	.2% TP	4	8	18.2	27.0	8.8 ± .312
7	.2% TH + .05TP	4	10	17.9	26.5	8.6 ± .349
<i>Exper. II. Maintained at 30°C.</i>						
8	Control	3	7	14.2	19.6	5.4 ± .301
9	.025% TP	3	3	14.2	16.6	2.4 ± .018
10	.2% TH	3	8	14.4	18.0	6.4 ± .338
<i>Exper. III. Maintained at 24°C.</i>						
1	Control	4	10	18.1	28.4	10.3 ± .286
2	.025% TP	4	10	18.4	30.2	11.8 ± .247
3	.05% TP	4	10	18.4	31.5	13.1 ± .386
<i>Exper. IV. Maintained at 30°C.</i>						
4	Control, 24°C.	4	5	16.9	25.6	8.7 ± .557
5	Control	4	6	17.0	24.3	7.3 ± .443
6	.005% TP	4	7	17.3	26.9	9.6 ± .297
7	.01% TP	4	10	17.3	24.6	7.3 ± .200
8	.02% TP	4	8	16.7	23.4	6.7 ± .239
9	.1% TH	4	7	16.7	22.9	6.2 ± .478

¹Thiouracil, ²Thyroprotein.

appearance of the mice were recorded daily between 8 A.M. and 9 A.M. The trials were continued for 4 weeks with the exception of *Experiment II*, which was terminated after 3 weeks. Details of the individual experiments are given in describing the results.

³A thyroactive iodinated protein, kindly supplied by the Cerophyl Laboratories, 2438 Broadway, Kansas City, Mo.

⁴Kindly supplied by the Lederle Laboratories, Pearl River, New York.

RESULTS

Body Weight. The essential details of the 4 experiments, including mice maintained at two different temperatures and receiving several different thyroid treatments are given in table 1. From the body weight data it is clearly demonstrated that the environmental temperature not only modifies the normal growth rate, but also influences markedly the thyroprotein tolerance level and the optimal range of dosage for growth stimulation. A rise of 6° in temperature from 24° to 30°C . re-

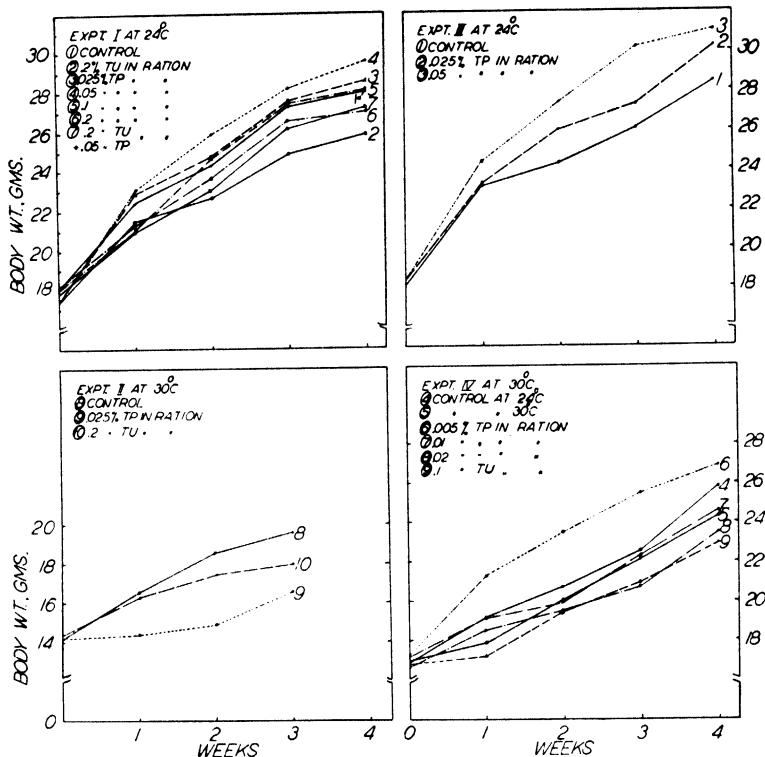


Fig. 1. EFFECTS OF THYROID STATUS on body growth of the male mouse at two different environmental temperatures.

sulted in a 1.4 gm. decrease in average body weight gains (*Exper. IV*). Although slightly below the 5 per cent level of statistical significance, this decrease is suggestive in that it is of similar magnitude as the highly significant decrease in weight gains of mice receiving 0.2 per cent thiouracil at 24°C . (*Exper. I*). At the higher temperature, 0.1 per cent thiouracil caused a still further reduction in the body weight gains.

At 24°C . highly significant growth stimulation was observed with 0.025 and 0.05 per cent of thyroprotein given in the feed (*Expers. I and III*). Adverse effects were noted in mice receiving more than 0.1 per cent thyroprotein. At 30°C ., a dosage

of only 0.025 per cent proved to be toxic (*Exper. II*), and the experiment was terminated after 3 weeks. On the greatly reduced level of 0.005 per cent thyroprotein in the ration (*Exper. IV*) growth was again stimulated so that the gains were significantly greater than those of the controls held at 30°C., and even exceeded slightly the gains of controls from the same lot of mice that were kept at 24°C. From the growth curves (fig. 1) it can be seen that the differences in rate of gain began to be evident by the first week of thyroprotein feeding and approached their maximum

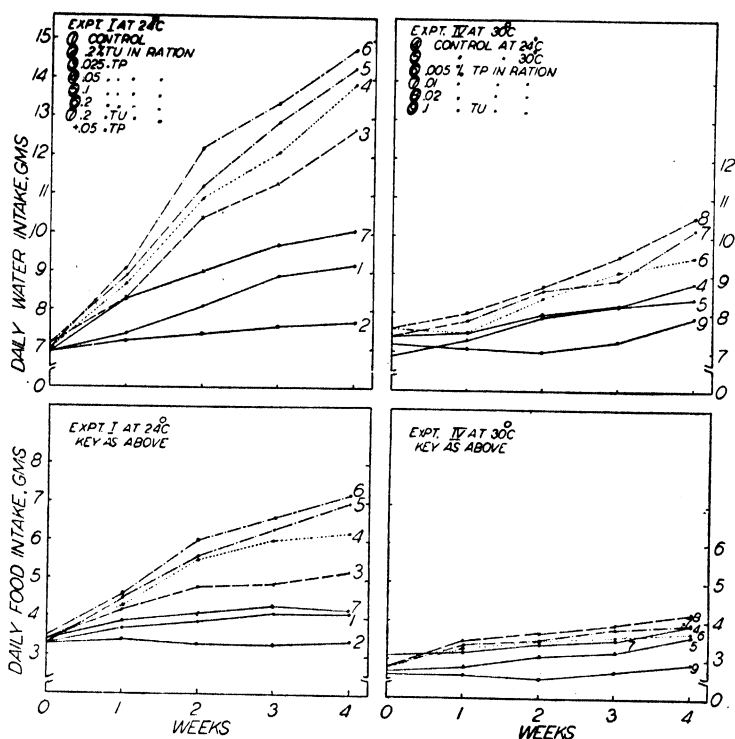


Fig. 2. INFLUENCE OF THYROID STATUS ON feed and water consumption of male mice at two different environmental temperatures.

by the end of the second week. It is also clearly shown that the optimum dosage of thyroprotein for growth stimulation of mice is decreased tenfold by simply increasing the environmental temperature by 6°C.

Feed and Water Consumption. Data on the feed and water intake were obtained only in *Experiment I* (24°C.) and *Experiment IV* (30°C.). Inasmuch as feed and water was supplied by groups, statistical treatment to determine the significance of differences between groups is not feasible. However, when the average daily feed and water intake per animal of each experimental group of mice is plotted against time in weeks, surprisingly close agreement is found between these measures and

the thyroid state (fig. 2). Thiouracil depressed the food intake by 15 per cent at both 24° and 30°C. Water intake was depressed to a greater extent at 30° than at 24°C. At both temperatures, groups of mice given progressively increasing amounts of thyroprotein increased their intake of food and water proportionate to the dosage. As indicated earlier, it was necessary to restrict the dosage at 30°C. to 0.02 per cent of thyroprotein or less because larger amounts were not tolerated. Nevertheless, the food and water intake showed good proportionality with dosage even in this restricted range, showing increases of 15.8 per cent and 15 per cent, respectively, at the highest level. The mice kept at 24°C. tolerated 10 times this maximum, the feed and water intake again increasing progressively with increasing dosage throughout the entire range. At the highest dosage level, the feed and water consumption were increased 56.4 per cent and 47.6 per cent, respectively.

DISCUSSION

It has been shown that the thyroid hormone secretion rate decreases normally with advancing age and at high environmental temperatures in several species of domestic animals (3, 4, 6-10). Hurst and Turner (4) state that stimulation of the growing mouse by 80 times its own thyroid secretion rate is detrimental to growth, but stimulating the growing mouse by 20 to 60 times its own thyroid secretion rate is beneficial to growth. This being so, the administration of small doses of thyroxine to growing animals will maintain an optimal thyroxine level in the body and will thus improve the metabolic rate within the physiological limits. Moreover, Evans *et al.* (11) and Scow and Marx (12) state that there are some indications that the thyroid hormone is necessary for the normal elaboration of the hypophyseal growth hormone.

Large doses of thyroprotein (0.2 per cent) in the feed markedly increased the feed and water consumption due to increased metabolic rate but there was a decrease in body weight gains. It may be pointed out here that increased feed consumption on high doses of thyroprotein treatment is in itself no criterion as to the increase in body weight. It has been observed that the mice with the highest feed consumption did not experience the maximum increase in weight. This suggests that there is a point of diminishing returns, wherein the increase in metabolism induced by the thyroprotein exhibits predominantly a 'catabolic' effect, in relation to the relative 'anabolic' effects which are produced by small doses of thyroprotein treatment in growing animals. There was a proportionately increased feed consumption with the increase in dosage of iodinated casein, but there was no direct relation as regards the increase in body weights.

The decrease in the thyroid function at high temperatures, and the accompanying reduction in feed intake and weight gains, are apparently one of the homeothermic mechanisms that enables the animal to maintain a relatively constant internal environment in the face of a changing external environment. It is of considerable interest, therefore, that even at 30°C. a low, and narrowly limited, amount of thyroidal stimulation is still capable of producing anabolic effects. It seems probable that the tolerance limits are determined principally by the temperature regulating capacity

since a tenfold increase in dosage is tolerated at an environmental temperature only 6°C. lower.

Data collected on the development of the sex organs of the mice involved in these experiments indicate that modifications in the thyroid status and environmental temperature can have a pronounced effect on sexual function as well as on body growth. In general, the conditions of hormone dosage and environmental temperature that were optimal for growth were also optimal for stimulation and maintenance of the testes and seminal vesicles. These findings will be published in detail in another paper.

SUMMARY

The results obtained in the present experiments indicate that mild hyperthyroidism stimulates while hypothyroidism depresses body weight gains in the growing male mouse at environmental temperatures of both 24° and 30°C. Thyroprotein when fed to growing male mice for a period of 4 weeks at 24°C. as 0.025 and 0.05 per cent of the ration caused highly significant increases in the body weight gains when compared with the control group. Control mice gained less at 30° than at 24°C. Mice fed 0.005 per cent thyroprotein for 4 weeks at 30°C. gained significantly more weight than the control group at 30°C. and slightly, but not significantly, more than the controls at 24°C.

Both feed and water consumption were depressed by either thiouracil feeding or elevation of the environmental temperature to 30°C. In mice fed graded amounts of thyroprotein, at both 24° and 30°C., the food and water intake increased proportionately with the thyroprotein dosage. An increase of only 6°C., i.e. from 24° to 30°C., in environmental temperature, caused a tenfold reduction in the optimal thyroprotein dosage in young male mice. Within a narrowly limited dosage range anabolic effects can still be observed at the higher temperature. It is suggested that under these experimental conditions the tolerance limits for thyroid stimulation are determined principally by the temperature regulating capacity.

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UTILIZATION OF PURE α -, γ -, AND δ -TOCOPHEROLS BY LAYING HENS¹

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THE non- α -tocopherols (β -, γ -, and δ -) are considerably less potent than α -tocopherol in their ability to prevent sterility and muscle dystrophy although they do not differ greatly in chemical structure. It has been suggested that what potency they do have is due to conversion to α -tocopherol. To test this possibility, hens maintained on a vitamin E-deficient diet were fed supplements of pure natural γ - or δ -tocopherol and their eggs were assayed to show whether or not the α -tocopherol content was increased. Information was also obtained regarding the relative deposition of α -, γ - and δ -tocopherol in hen's eggs.

METHODS

Mature hens were kept in individual cages in air-conditioned rooms (75° F.; 50% R.H.) and fed a commercial laying mash until a regular egg production pattern was established. They were then transferred gradually over a period of 10 days to a vitamin E-deficient diet, similar to that of Dam (1), table 1.

On this diet hens showed markedly decreased egg production or stopped laying altogether. Supplements of the various pure, natural tocopherols² were given in capsules, usually 5 per week. The dosage level varied from 100 to 4000 mg/week, each level being fed for 2 to 4 weeks.

Eggs were collected and stored in the deep-freeze at -22°C. until time of analysis. They were analyzed for total and for γ -plus δ -tocopherols, α -tocopherol being calculated by difference. Tocopherol analyses were made on occasional samples of blood and feces.

The assay methods for total and for γ -plus δ -tocopherols in eggs consisted primarily of the steps described for analysis of foods for vitamin E (3). However, 2 extraction techniques were compared: 1) extraction with a 1:1 mixture of chloroform and ethanol, followed by filtration, evaporation and re-extraction of the residue with chloroform, and 2) extraction with a mixture of 50 per cent ethanol and petroleum ether. Both procedures extracted more carotene than could be conveniently decolorized in the semi-micro hydrogenator. Accordingly, weighed portions of the lipid extract from eggs were molecularly distilled, thus effecting a separation of part of the carotene from the tocopherols. The distillate containing the tocopherols was then hydrogenated and assayed photometrically for tocopherols.

Extraction according to the first procedure always gave more lipid and usually

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² Kindly supplied by the Organic Research Dept. of Distillation Products, Inc.

more tocopherol than did extraction according to method 2). The maximum difference was about 25 per cent. However, the proportion of γ - and δ - to total-tocopherols was the same with both techniques. Since only comparative tocopherol values in eggs were wanted, the simpler alcohol-petroleum ether extraction procedure was used.

In this, 8 gm. of well-mixed whole egg + 8 ml. of distilled water + 16 ml. of absolute ethanol + 40 ml. of purified Skellysolve B in a glass-stoppered cylinder were shaken for 10 minutes. Twenty milliliters of the supernatant were evaporated to dryness under nitrogen in the aluminum cup which lines the molecular still. The lipid residue was weighed, and then distilled, hydrogenated, and assayed by photometric methods for tocopherol content.

TABLE 1. COMPOSITION OF DIET NO. 300¹

<i>Ingredient</i>	<i>%</i>
Casein, crude.....	15.0
Dried yeast.....	10.0
Corn starch.....	50.8
Gelatine.....	8.0
Gum arabic.....	4.0
Salt mixture ²	7.0
L-cystine.....	0.1
Choline chloride.....	0.1
Vitamin K (10 mg/kg.).....	—
Cod liver oil (Added fresh daily to mixture of dry ingredients).....	5.0
	<hr/> 100.0

¹ The vitamin E-free diet assayed 1.67 mg/100 gm of total tocopherols and 0.19 mg/100 gm of γ - plus δ -tocopherols. However, these results were probably caused by a tocopherol-like substance contained in the yeast of the diet. Such a material has been studied. It is fat-soluble, non-saponifiable, Emmerie & Engel (2) positive before and after hydrogenation, and it couples with diazotized di-anisidine. However, it shows no biopotency in rat anti-sterility assays. Hence, the diet can be considered virtually vitamin E-free.

² Salt mixture: U.S.P. No. 2 salt mixture, 348 gm., plus manganese sulfate, 2 gm.

All egg assays were done at least in duplicate and usually in quadruplicate. Replicate assays showed excellent agreement.

RESULTS

In a preliminary experiment, 3 hens were used. These produced eggs with total tocopherol content of 2.5 to 4 mg/100 gm. of whole egg. This range of values decreased to 0.4 to 1.0 mg. after 2 weeks of depletion during which the vitamin E-free diet No. 300 was fed. Weekly supplementation of these hens with 100 mg. of d, α -tocopherol caused the total tocopherol content of eggs to increase to 4 to 15 mg/100 gm. However, γ -tocopherol supplementation at the same level resulted in no increase in egg tocopherol concentration. Higher levels of γ -tocopherol supplementation were necessary to produce eggs with enhanced total tocopherol content. For example, a hen, which laid eggs with 9.00 mg. of tocopherol/100 gm. after supple-

mentation with 100 mg/week of α -tocopherol, laid eggs with only 2.5 mg. of tocopherol/100 gm. when fed 500 mg. of γ -tocopherol per week.

The extra tocopherol, deposited in eggs as a result of high-level γ -tocopherol supplement to the hens' diet, appeared to be chiefly γ -tocopherol.

To confirm and extend these observations a second experiment was made. Laying hens, weighing between 3.5 and 5 lb., were maintained on laying mash for one week and then shifted gradually (10% each day) to the vitamin E-free diet No. 300. During the first week, average values were 0.88 to 1.53 mg. of total tocopherols/100 gm. and 0.12 to 0.30 mg. of γ - plus δ -tocopherols, respectively. Thus, eggs produced by hens on a presumably normal laying ration contained tocopherols, approximately 85 per cent of which was α -tocopherol.

During the conversion from commercial mash to vitamin E-free diet there was little change in the tocopherol content of the eggs. However, after the hens had been on diet No. 300 for a week, the tocopherol content of all the eggs was definitely lower, e.g. 0.16 to 0.64 mg/100 gm., and practically 100 per cent of this vitamin E was α -tocopherol.

After several weeks on the vitamin E-free diet, the hens showed changes in egg production. For the first 5 weeks they laid eggs at a constant and characteristic rate (4 to 6/week). At 8 and 9 weeks the rate dropped to 1 or 0/week for each of the hens. At this time the hens had blood vitamin-E levels of less than 0.1 mg/100 ml.

Supplements of the various pure tocopherols were started at the eighth or ninth week. Egg production increased immediately, sometimes to pre-depletion levels, although the rate fluctuated depending upon the level of α -tocopherol supplementation.

No evidence was found in this experiment to show that there is conversion of non- α - to α -tocopherol. Hens fed δ - or γ -tocopherol laid eggs with increasing proportion of non- α -tocopherol, i.e. up to 90% of total, as the level of supplementation was increased.

The prompt and steep increase in amount of tocopherol deposited in eggs of hens which received α -tocopherol is in marked contrast to the slow and slight increase of tocopherol in eggs laid by hens on γ - or δ -tocopherol supplementation. This is evident from figure 1 in which concentration of total tocopherols in eggs as a function of log dose (weekly supplement) is plotted. The values for egg tocopherol concentration are those found in the second week of supplementation of hens at the given level; limited supplies of the pure tocopherols precluded longer supplementation at every level. However, change in egg tocopherol concentration between the second and third weeks was slight, compared to that between the first and second week of hens on supplement. Tocopherol concentration in eggs increased proportionally to the log-dose (fig. 1). The slopes of the curves are 31, 7, and 3 for α -, γ -, and δ -tocopherols, respectively. Consequently, γ -tocopherol was only about one fourth as effective as α -tocopherol and δ -tocopherol only about one tenth as effective as α -tocopherol in these experiments.

This relation between amounts of the pure tocopherols laid down in eggs is consistent with the relative physiological activity of the tocopherols in relieving a variety of deficiency syndromes in several species. It is similar to data on relative

amounts of deposition of various tocopherols in body tissues of rats (4). Studies in our laboratory have shown that cows fed equal amounts of α - or γ - plus δ -tocopherols secrete proportionately much more of the former than the latter in milk (5). Assays of human tissues and calculations of dietary intake of the various forms of tocopherol suggest a similar relation for deposition of ingested tocopherol in the body tissues of humans (6).

The efficiency of transfer of tocopherols to eggs reached a maximum in these experiments at the lowest levels fed (100 to 200 mg/week). This efficiency is expressed as follows:

$$\% \text{ efficiency} = 100 \times \frac{\text{total tocopherol in eggs laid/week}}{\text{amount of tocopherol fed/week}}$$

Typical efficiency values for α -, γ -, and δ -tocopherol-fed hens were, respectively, 16.5, 2.9, and 1.4 per cent.

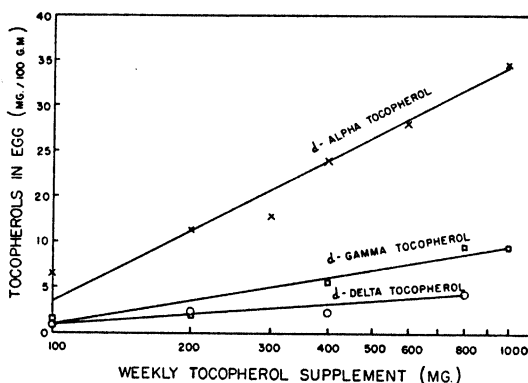


Fig. 1. TOCOPHEROL CONCENTRATION in hens' eggs (2nd week of pure tocopherol supplement).

Preliminary data regarding the vitamin-E requirement of laying hens may be drawn from these experiments. The laying mash contained, by chemical assay, 6.29 mg. total tocopherols/100 gm. Because of interfering materials, non- α -tocopherols could not be assayed chemically. They may be assumed to comprise one half of the total, since bioassays of a wide variety of grains in our laboratory have shown that about 50 per cent of total tocopherols are non- α -tocopherols. If a hen consumed 100 gm/day of mash, about 3 mg. of α -tocopherol/day would be derived from the diet. On the average, each hen laid 5 eggs/week, each weighing about 55 gm., or about 275 gm. of egg/week. Eggs laid during this period averaged about 1.2 mg. of α -tocopherol/100 gm. Thus, of 21 mg. of α -tocopherol which the hens received from the diet, they put out $(275 \times 1.2/100)$ 3.30 mg. in eggs. The efficiency of transfer $(3.30/21 \times 100 = 15.7\%)$ is about equal to that calculated for α -tocopherol-fed hens at the lower levels of supplementation in these experiments.

Thus, a daily intake of 3 mg. α -tocopherol by the hen permitted egg production with higher than minimal α -tocopherol content, normal metabolic processes, and

probably some storage of α -tocopherol since 8 to 9 weeks were required to deplete these hens of stored tocopherols. Assuming a weekly output of 5 eggs, a minimum concentration of 0.5 mg. tocopherol/100 gm. of egg, and 55 gm. the average fresh weight of eggs, then $\frac{5 \times 0.5 \times 55}{100} = 1.37$ mg. of tocopherol, the minimal weekly output of tocopherol. The minimal weekly intake would then be $(100\%/16\% \times 1.37$ mg.) 8.6 mg. of α -tocopherol or about 1.2 mg/day which might be considered the minimum requirement.

Blood levels of vitamin E were determined on several of the hens on various tocopherol supplements. After one week of supplementation with 1600 and 2000 mg. of α -tocopherol, respectively, 2 hens had plasma vitamin-E levels of 20.0 and 20.1 mg/100 ml. In contrast, a hen on 1000 mg/week of γ -tocopherol had 2.1 mg. per cent tocopherols in blood, and a hen which received 800 mg/week of γ -tocopherol had 1.35 mg. per cent tocopherols. It is evident that blood tocopherol levels of hens, like the amounts in their eggs, are much higher following α -tocopherol than γ - or δ -tocopherol supplementation.

Occasional fecal samples from the hens were assayed for total tocopherols and for individual tocopherols by both dianisidine-coupling (7) and nitroso-assay methods (8). In all cases the preponderant form of tocopherol excreted was the same as that fed. No conversion of one form of tocopherol to another was indicated. One of the hens which was fed one gm/day of α -tocopherol excreted 770 mg. in a 24-hour fecal sample.

SUMMARY

Laying hens, maintained on a vitamin E-free diet, were given supplements of pure α -, γ -, or δ -tocopherols at levels of 100 up to 4000 mg/week. Their eggs were assayed for total and for γ - + δ -tocopherol content. α -Tocopherol content was estimated by difference. No evidence of conversion of γ - or δ -tocopherol to α -tocopherol by the hen was found. The tocopherol concentration of the eggs varied linearly with the log-dose. The α -tocopherol induced the greatest deposition; γ -tocopherol was one fourth and δ -tocopherol was one tenth as effective as α -tocopherol. From these preliminary data, it is calculated that the laying hen requires a minimum of about 1.2 mg/day of α -tocopherol. Large amounts of unchanged tocopherol were excreted in the feces of hens on high level supplements of various pure tocopherols. Blood-tocopherol levels of hens on α -tocopherol supplement were several-fold greater than those of hens receiving similar amounts of γ - or δ -tocopherols.

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EFFECTS OF SOME ION EXCHANGE RESINS ON THE MINERAL METABOLISM OF RATS

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THE suggestion that ion exchange resins might be used for the purpose of decreasing the extent of absorption of electrolytes, particularly sodium ions, was first published by Dock (1). This treatment would represent a very valuable addition to the armamentarium of the physician for the management of cases such as those of congestive heart failure and hypertension, in which the retention of sodium leads to edema. In the past such cases have been treated chiefly with mercurial diuretics and by severe restriction of salt intake. That the latter procedure is one which must be used with care has been emphasized by Soloff and Zatuchini (2). Clinical trials of ion exchange resins have been made with generally favorable (3), or partly favorable results (4, 5). This approach has the obvious advantage that sodium absorption can be limited while allowing the patient a more nearly normal diet, or this form of treatment can be used to reinforce other not completely adequate measures.

For testing purposes laboratory animals are ideal since they will freely consume a diet of exactly known composition, and the excreta are readily collected quantitatively. This permits a rapid and accurate evaluation of the ion exchange materials currently available, as well as combinations of them. Such an evaluation should have the ultimate objective of blocking the absorption of sodium as completely as possible with the smallest bulk of resin, and to do so with a minimal disturbance of acid-base balance.

Thus far data on the effects of ion exchange resins in laboratory animals are meager. Dock (1) reported evidence that Ionac C-284 decreases sodium absorption in rats, but did not give detailed experimental data. More recently Crismon (6) presented a report on the effects of feeding Dowex 50 at a 10 per cent dietary level to rats. Sodium absorption was definitely decreased, but potassium absorption was relatively unaffected. After 19 days on this regime the animals were autopsied; plasma sodium and potassium were essentially normal, but in the muscle, sodium, potassium and chloride were low.

A wide variety of excellent ion exchange materials is now available (7). There are cationic exchange resins of the sulfonic acid type and fairly high capacity, and carboxylic acid exchangers of very high capacity. Basic, or anionic, exchangers of both intermediate and weak basicity are available, both with high capacities. However, at pH 6 to 7, the usual reaction of the intestinal contents, the available capacities of both the carboxylic and the weakly basic exchangers are much reduced (7).

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An increase in pH would increase the capacity of the carboxylic resin, while decreasing the capacity of the weakly basic resin, and vice versa. On the other hand, a basic resin of intermediate strength would be expected to retain most of its capacity, even at neutrality.

The purpose of this study has been to determine which of the presently available ion exchangers, or combinations thereof, represents the most efficient means of binding sodium in the digestive tract, while at the same time binding the least possible amounts of potassium and calcium. As pointed out above, it would also be desirable to make some provision for the maintenance of acid-base balance. It is not to be expected that any of the ion exchangers will react to their full capacity in the digestive tract since the full capacity is only available as a result of prolonged exposure to an excess of the ion to be exchanged and furthermore, the diffusion of ions toward the adsorbing surface is naturally impeded by the heterogeneous and viscous character of the medium.

EXPERIMENTAL

Ion Exchange Materials. The following resins have been tested in these experiments:

NAME	TYPE	CYCLE IN WHICH USED	CLAIMED CAPACITY ¹ mEq/gm.
Amberlite XE-58 ²	Weak base	OH ⁻	10.0
Amberlite XE-64 ²	Carboxylic	H ⁺ , K ⁺	10.0
Amberlite XE-66 ²	Sulfonic acid	H ⁺	4.2
Permutit XAC ³	Carboxylic	H ⁺	10.0
Ionac A-300	Intermediate base	OH ⁻	7.3
Win 3000	Sulfonic acid	NH ₄ ⁺	5.0

All of these were administered in a particle size of 60 to 80 mesh, thoroughly mixed with the food at definite levels.

Methods for Metabolic Studies. The experimental subjects were 5 pairs of rats weighing initially 215 to 270 gm. They were kept in 5 metabolism cages fitted with stainless steel funnels; urine was collected in paraffin-lined bottles using thymol as a preservative, and feces were collected on a wire screen. Food and water were provided *ad libitum*, with account being kept of the consumption of both. The various dietary regimes were given for 6-day periods but the excreta were pooled for analysis for 3-day periods. This was done in order to show data on the rate at which equilibrium is established following a change of diet. At the end of each period the collecting funnel was washed down with water, any scattered food was collected and dried at 100°C., and its weight was subtracted from the gross food consumption. Feces were thoroughly dried at 100°C., then ground and mixed before aliquots were taken for analysis.

Analytical Methods. Urine samples were diluted to 200 cc., and further dilutions of these were analyzed for sodium and potassium in a Model 52A Perkin-Elmer flame photometer, using the internal standard method. Calcium was determined on an aliquot of urine by a procedure which was essentially that of Puppel and Curtis (8). Chlorides were determined by the method of Schales and Schales (9), using a slight excess of nitric acid.

¹ By the manufacturer.

² Kindly supplied to us through the courtesy of Dr. James C. Winters of Rohm and Haas Company, Philadelphia. These products are essentially the same in properties as Amberlite IR-4B, IRC-50, and IR-120, respectively.

³ Kindly supplied to us through the courtesy of Mr. M. E. Gilwood of Permutit Company, New York.

Feces were prepared for analysis as directed by Hald (10). The ash from 2-gm. samples was dissolved in dilute HCl, and further dilutions of this were analyzed for sodium and potassium in the flame photometer. Calcium was determined on an aliquot of the same ash solution by precipitation as the oxalate at pH 5, and titration with permanganate according to Tisdall and Kramer (11). Chlorides were determined in feces by the open Carius method (12). Samples of serum were analyzed for sodium and potassium by the methods of Hald, and muscle samples were prepared by the same procedure as used for feces. Food samples were also analyzed by the methods mentioned above.

The basal diet used during the first two periods (12 days) contained,⁴ as mEq/100 gm.: Na, 30; K, 20.2; Ca, 26.5; and Cl, 32.4. For the remaining periods (24 days) only one per cent of NaCl was added to the diet, giving this composition as mEq/100 gm.: Na, 19.6; K, 20.2; Ca, 26.5; and Cl, 20. The composition of each individual resin diet was checked by analysis; it was as expected from the particular dilution of the above amounts, with these exceptions: 1) extra potassium was present when the resin partly was added in the potassium cycle, 2) Amberlite XE-66 was furnished to us in the sodium cycle; after washing with acid and water and drying it still contained 0.77 per cent sodium, and 0.20 per cent chloride, and 3) Ionac A-300, which had been used for other purposes and regenerated, contained 2.55 per cent chloride, about 11 per cent of its capacity.

Screening Experiments. In the preliminary experiments the test periods were kept relatively short. This was done so that the largest possible number of comparisons of the various resins could be made, to establish tentatively the most efficient regimes for sodium diversion. From data on intake and output, balances were calculated for sodium, potassium, chloride and water. The amounts of the bases bound per gram resin ingested were also calculated, and may be taken as the best index of efficiency. The results of these screening experiments are given in a somewhat simplified form in table 1; the experimental protocols are too extensive to be given in their entirety.

Long-term Feeding Experiment. Since the above data were obtained on diets having high mineral contents, with short feeding periods, it was decided to carry out a long-term experiment on a low-sodium diet to determine whether an actual depletion of the body stores of sodium is possible by the ion exchange method. There is already some evidence (table 1: *pair A*, period 4; *pair D*, period 3) that such depletion can occur. However, if even 10 per cent of the dietary sodium is absorbed at a level of 20 mEq./100 gm. of food, enough is available to the animal to ensure a positive sodium balance (13); depletion would require more drastic conditions. Accordingly, a basal diet was prepared⁵ which gave the following analysis as mEq./100 gm.: Na, 2.3; K, 12.2; Ca, 37; and Cl, 3.4. The feeding experiment was conducted along the lines described above with the exception that the dietary regime of each pair of animals remained the same for 21 consecutive days. New rats were used, and the resin combinations were those which were considered the most effective as shown by the screening experiments. All resins, or combinations, were fed at a 10 per cent level, and one control pair received the basal diet without resin. Excreta were collected frequently, but were pooled for weekly periods for analysis. On the twenty-first day blood samples were taken by heart puncture, and the serum was separated. The animals were then killed, and samples of the *vastus lateralis* muscle

⁴ Percentage composition: ground yellow corn, 28.1; ground whole wheat, 28.1; skimmed milk powder, 22.8; linseed meal, 8.0; alfalfa, 3.0; commercial casein 4.1; calcium carbonate, 0.5; sodium chloride, 1.5; yeast, 3.0; and cod liver oil, 0.9.

⁵ Percentage composition: ground yellow corn, 28.3; ground whole wheat, 28.3; commercial casein 26.7; linseed meal, 8.2; alfalfa, 3.0; calcium carbonate, 1.7; yeast, 3.0; and cod liver oil, 0.9.

were taken for analysis. Sodium and potassium were determined on both serum and muscle. The results of this experiment are presented in table 2.

Chronic Toxicity of Ion Exchange Resins. Four groups of 10 young rats weighing initially 55 to 65 gm. each, and about equally divided between males and females, were subjects for the chronic toxicity test. One group served as controls, and the other groups received in their diets⁶ sulfonic acid resins of the Win-3000 type at a 10 per cent level. To one group the resin was fed in the hydrogen cycle, to another in the sodium cycle, and to the third in the ammonium cycle. The feeding experiment was continued for 60 days, with frequent recording of body weights. All of the animals survived, and the growth patterns of the several groups were practically identical. The final growth percentages were as follows: hydrogen cycle, 312; ammonium cycle, 315; controls, 330; and sodium cycle, 365. These differences are not considered to be of statistical significance. At necropsy no pathological changes were found in the gastrointestinal tract of any of the animals.⁷

While no study of the effects of ion exchange resins on the absorption of the B vitamins has been included in this work, it may be deduced from the above growth experiments that, although the resins may have bound some of the vitamins in the food, the amounts which escaped binding were entirely adequate for normal nutrition.

DISCUSSION

All of the cationic exchange resins tested had a definitely positive effect as regards the binding of sodium and potassium in the gastrointestinal tract. The amounts of these elements bound per gram resin appeared to be independent of the level at which at least one of the resins (Win 3000) was fed. The amount of sodium bound per gram resin was also not appreciably affected by reducing the sodium content of the basal diet from 30 to 20 mEq/100 gm., but it was markedly affected when the sodium content was reduced to 2.3 mEq/100 gm. (see table 2). Some of the resins bound rather large amounts of calcium, but this did not appear to be true of the one which was fed in the ammonium cycle. The natural variability of calcium absorption from group to group, and from one period to another (see below), makes it difficult to arrive at definite conclusions on this point. However, if the indicated conclusion is correct, the most likely explanation appears to be that when the resins are fed in the hydrogen cycle, the hydrogen ions released in the exchange reaction favor the solution of calcium salts.

Water balances were high when the amount of sodium absorbed was high, and were low when most of the sodium was bound, as would be expected.

The manner of calculating the amounts of the elements bound by the resins requires detailed comment. It cannot be assumed that all the sodium and potassium found in the feces on the resin diets is bound to resin, since allowance must be made for fairly large amounts of these elements which appear in the feces on the control diet. In the case of sodium and potassium, about 25 per cent remains unabsorbed on the control, or basal diet, while for calcium and chloride the corresponding figures

⁶ Basal diet had same composition as that given in footnote (4) except that NaCl content was one per cent.

⁷ Gross and histopathological examinations were made by Dr. F. C. Goble.

TABLE 1. EFFECTS OF SOME ION EXCHANGE RESINS ON MINERAL AND WATER METABOLISM OF RATS ON DIETS OF RELATIVELY HIGH MINERAL CONTENT

PERIOD ¹	AV. INITIAL WT.	RESIN ADDED TO DIET	% ADDED	WATER BAL-ANCE	RESIN IN-GESTED	SODIUM METABOLISM				POTASSIUM METABOLISM				CALCIUM METABOLISM				CHLORIDE METABOLISM			
						Inake	Unab-sorbed	Re-tained	Bound by resin	Inake	Unab-sorbed	%	Bound by resin	Inake	Unab-sorbed	%	Bound by resin	Inake	Unab-sorbed	%	Retained
Pair A																					
1 ^a	250	none	6}	171	185	29.9	14.2	+21.4	20.1	22.9	-18.2	26.3	58.5	+40.1	32.2	7.1	+23.5	32.2	7.1	+23.5	
						31.5	22.5	-2.6	21.2	32.8	-8.2	27.8	76.2	22.7	34.0	11.4	+4.8	34.0	11.4	+4.8	
2	266	Ambit. XE 64 Ambit. XE 58	4}	200	107	32.3	42.2	+7.1	1.07	21.4	40.6	-3.5	0.37	28.5	70.8	10.8	0.51	35.0	15.3	+13.8	
						32.3	50.7	-1.3	1.40	21.4	51.2	-9.3	0.68	28.5	82.8	16.3	0.63	35.0	21.1	+2.0	
3	277	none		108	169	31.8	16.5	+2.6	32.9	30.1	+25.2	43.1	52.0	46.0	32.2	9.9	+4.5	32.2	9.9	+4.5	
						27.3	20.6	+9.1	28.3	26.1	+13.0	37.0	74.3	21.7	27.6	12.4	+4.2	27.6	12.4	+4.2	
4	328	Ambit. XE 66	10	167	151	28.8	46.0	+14.3	0.57	24.6	45.3	+7.0	0.31	32.8	85.2	13.1	0.53	25.3	21.7	+5.1	
						25.2	71.0	-11.3	1.10	21.5	57.8	-13.0	0.54	28.6	93.2	4.9	0.70	22.0	32.4	-21.2	
5	318	none		212	146	25.6	25.2	+16.3	26.5	32.3	+11.7	34.7	62.0	36.1	25.9	5.7	+7.8	25.9	5.7	+7.8	
						21.7	27.3	+13.8	21.0	31.3	+2.8	29.4	78.9	19.9	21.8	21.0	+7.0	21.8	21.0	+7.0	
6	338 339 ⁴	Win 3000	10	203	158	15.2	43.6	+29.8	0.33	15.6	42.4	+6.2	0.20	20.5	77.5	20.7	0.18	15.3	4.1	+21.8	
						13.2	59.8	+9.6	0.60	13.6	39.8	+11.5	0.15	17.9	70.7	26.5	0	13.4	8.9	+2.7	
Pair B																					
1	244	none		198	176	29.9	17.3	+24.1	20.1	24.1	-18.8	26.3	65.3	33.2	32.2	9.9	+25.5	32.2	9.9	+25.5	
						39.1	15.8	+21.2	26.3	24.5	+16.2	34.4	56.5	42.2	42.2	9.7	+21.9	42.2	9.7	+21.9	
2	291	Ambit. XE 64	10	177	185	29.0	48.5	+10.0	0.86	19.2	47.4	-2.7	0.41	25.7	73.3	25.3	0.25	31.4	12.6	+11.7	
						32.1	75.4	+3.5	1.61	21.3	69.0	-0.6	0.82	28.2	90.5	8.7	0.70	34.6	17.1	+9.6	
3	308	Ambit. XE 64, H Ambit. XE 64, K	8.7 1.3}	165	172	28.8	62.5	+4.4	0.66	44.6	59.6	+10.2	1.21	39.0	66.0	33.1	0.07	29.0	10.9	+4.2	
						27.4	69.0	+8.2	0.77	38.4	50.0	+5.2	0.92	33.5	89.6	9.1	0.65	24.9	15.5	-0.3	
4	343	none		164	191	27.5	29.1	+4.9	28.4	30.0	+12.7	39.3	55.8	42.3	27.8	9.2	+15.6	27.8	9.2	+15.6	
						22.4	21.0	+12.6	23.1	26.0	+8.3	30.3	72.4	26.1	22.6	11.5	+10.1	22.6	11.5	+10.1	

5	356	Permutit XAC	10	172	12.9	22.7	45.0	+15.4	0.35	23.5	41.3	+10.7	0.24	30.8	85.7	13.0	0.41	23.0	8.9	+21.7
6	353	none	100	148	11.7	20.6	44.6	+9.1	0.33	21.2	37.7	+10.1	0.18	27.9	84.0	14.8	0.50	20.8	11.8	+12.2
368	189			19.4	17.0	+30.1	20.0	23.1	+21.4	26.2	60.6	38.8	10.6	6.6	+25.5	19.9	9.8	+7.3		
Pair C																				
1	231	none	6.7 3.3	157	20.9	14.2	+21.7	20.1	21.2	-20.1	26.3	58.4	39.6	32.2	8.6	+20.0				
2	266	Ambt. XE 64 ^s Ionac A-300		200	33.0	20.6	+7.4	22.2	26.9	+7.5	29.1	60.0	38.3	35.6	12.5	+14.3				
3	303 ^s	none	10	128	8.2	22.0	55.3	-7.9	1.52	14.7	53.9	-25.0	0.53	19.5	92.0	7.2	1.17	25.8	13.4	+22.6
4	335	Ambt. XE 64		102	16.2	68.8	-8.1	2.07	10.7	52.2	-14.6	0.50	14.2	87.4	11.2	0.98	18.9	11.3	+15.4	
5	333	none	9.35 0.65	193	24.6	25.1	+10.6	25.4	24.4	+36.1	33.2	58.3	40.2	24.7	11.0	+14.8				
6	331	Ambt. XE 64, H		187	26.1	45.9	+15.0	27.0	28.6	+23.4	35.3	67.8	30.8	26.3	17.7	+8.7				
7	333	none	10	145	10.4	18.4	58.2	+10.2	0.41	18.7	53.3	-0.4	0.35	24.9	97.0	1.2	0.82	18.5	20.7	+7.6
8	331	Ambt. XE 64, K		226	21.0	88.2	-13.5	1.12	21.4	56.3	+12.0	0.41	28.3	83.4	15.3	0.48	21.2	21.7	+3.8	
9	333	none	9.35 0.65	181	26.5	24.0	+23.9	27.4	28.1	+28.2	35.6	55.0	43.0	26.8	7.5	+31.7				
10	331	Ambt. XE 64, H		147	21.3	27.7	+12.3	22.0	27.9	+11.4	28.7	73.9	25.2	21.4	17.0	+12.0				
11	333	Ambt. XE 64, K	9.35 0.65	223	10.9	19.2	35.4	+44.6	0.19	24.9	38.2	+18.6	0.42	26.0	72.0	27.1	0.21	10.4	11.4	+24.7
12	333	Ambt. XE 64, K		207	11.3	20.0	71.4	+7.8	0.48	25.8	52.5	+11.0	0.74	27.0	88.5	10.2	0.62	20.2	20.3	+19.6
Pair D																				
13	221	Win 3000	10	141	9.5	25.8	41.0	+16.7	0.65	17.1	43.5	-22.0	0.35	22.7	79.0	40.2	0.25	27.8	13.8	+13.5
14	237	none		140	11.2	29.8	39.0	+28.4	0.59	20.1	46.5	+17.1	0.41	26.7	66.1	33.5	0	32.7	12.0	+34.8
15	264	Win 3000	15	158	32.4	16.3	+21.0	21.8	21.1	+21.6	28.6	61.8	37.0	35.0	7.8	+26.9				
16	266	none		146	32.0	17.0	+21.0	21.6	23.3	+17.4	28.2	73.3	24.7	34.5	11.7	+22.8				
17	264	Win 3000	15	95	12.5	13.9	87.0	-18.5	0.59	17.1	100.0	-29.5	0.81	18.7	82.4	16.6	0.19	13.6	13.3	-17.0
18	266	none		111	17.6	19.5	90.5	-16.4	0.64	20.1	53.2	+23.9	0.27	26.4	73.5	25.1	0	19.6	5.8	-5.3
19	272	Win 3000	10	151	23.2	28.8	+23.0	24.0	31.2	+31.2	31.4	65.3	32.0	23.4	10.2	+14.0				
20	289	Ambt. XE 64, H		164	21.2	22.5	+0.8	21.9	27.1	+2.9	28.5	79.6	18.2	21.3	13.5	-5.2				
21	272	Win 3000	10	140	10.6	18.6	57.5	+21.4	0.56	10.3	50.6	+14.1	0.39	25.3	63.6	34.2	-0.21	18.8	10.6	+34.6
22	289	Ambt. XE 64, K		139	10.3	18.2	63.6	+8.4	0.67	18.9	45.3	+13.7	0.30	24.0	67.8	27.8	0	18.4	9.4	+19.3
23	289	Ambt. XE 64, H	8.05 1.95	177	10.5	18.5	49.3	+23.8	0.42	33.9	35.1	+23.9	0.67	25.0	77.8	20.6	0.24	18.6	10.7	+24.7
24	399	Ambt. XE 64, K		184	11.7	20.6	72.9	+5.5	0.84	37.8	39.1	+15.3	0.79	27.9	70.8	12.9	0.33	20.8	19.1	+16.8

Sodium														
In food, mg.	112	86	63	124	119	135	102	115	121	90	121	133	116	117
In urine, "	48	84	28	20	20	10	15	5	10	20	5	10	20	18
In feces, "	18	4	6	04	120	108	82	103	81	50	84	90	66	90
Balance	+46	-2	+20	+2	-21	+16	+5	+7	+30	+28	+32	+33	+30	0
Bound by resin mEq/gm. ¹				0.14	0.19	0.15	0.14	0.20	0.12	0.08	0.13	0.12	0.14	0.23
Potassium														
In food, mg.	1020	783	532	1140	1090	1155	933	957	1040	1600	1895	2000	1060	1068
In urine, "	635	625	402	105	66	70	158	55	70	445	386	420	217	62
In feces, "	130	46	61	837	907	961	737	814	884	1036	1203	1355	793	885
Balance	+255	+112	+10	+108	+117	+124	+38	+88	+86	+167	+267	+225	+50	+121
Bound by resin mEq/gm.				0.66	0.76	0.76	0.72	0.78	0.78	1.11 ⁷	1.15	1.15	1.00	1.13
Calcium, mg.														
In food	1595	1222	828	1765	1700	1700	1418	1402	1010	1408	1578	1770	1650	1662
In urine	30	23	19	11	11	11	10	7	12	10	8	10	11	9
In feces	1430	655	810	1445	1470	1060	1180	1321	1470	1270	1462	1600	1286	1372
Balance	+135	+544	-1	+309	+219	+119	+258	+164	+128	+128	+168	+160	+360	+281
Chloride, mg.														
In food	258	168	134	286	276	290	234	242	262	228	267	287	469 ⁸	475
In urine	183	167	81	263	246	236	209	183	168	196	137	165	367	366
In feces	37	5	13	47	20	16	38	14	27	31	18	33	80	69
Balance	+38	+26	+40	-24	+10	+38	-13	+47	+67	+1	+92	+89	+22	+40
Blood Serum, ¹⁰ mg. %														
Sodium	310			330, 330			334, 338		316, 316		332, 320		344, 326	
Potassium	23.7			11, 23.7			22.0, 24.0		25.5, 25.2		23.2, 23.7		23.5, 11	
Dry Fat-free Muscle, ¹⁰ mEq/100 gm. ¹¹														
Sodium	11.0			8.9			9.5		9.5		10.3		10.0	
Potassium	44.0			43.0			42.3		41.1		42.7		40.1	

¹ Total resin in diet was 10%. ² 1.35% in K cycle, 8.65% in H cycle. ³ 6.7% of Amberlite XE-64, 3.3% of Ionac A-300. ⁴ One rat in this group became ill about the 12th day and died on the 17th, as a result of respiratory infection. Weight figures are given only for the surviving animal, also metabolic data for the third week are for this animal only. ⁵ Na content of diet 0.106%, chloride content 0.126%, since traces of these elements were present in the resin. ⁶ For method of calculation, see Discussion. ⁷ In arriving at this figure correction for normally unabsorbed K is made only for that part of the K which is present in the food, and a similar consideration applies with respect to Na in group 6. ⁸ Chloride content of diet, 0.19%. ⁹ Chloride content of diet, 0.26%. ¹⁰ On 21st day. ¹¹ Sample not sufficient for K analysis. ¹² Analysis was made on equal wgt. of muscle from each rat of pair. Muscle was dried overnight at 100°C., was then powdered and extracted 3 times each with acetone and ether, and finally dried in vacuo before taking sample.

are 63 and 10, respectively. This is a larger amount of sodium and potassium than is usually reported to be in the feces (13). It probably results from the high percentage of whole grains in the diet. Therefore, in calculating the amounts of the various elements bound by the resins, correction must be made for the average percentage of these elements which remain unabsorbed in the most comparable control experiment in the same pair of animals. The method of calculation is as follows:

mEq. bound per gm. resin = $\frac{(X - Y)Z}{100a}$, where X is the percentage of ingested Na, K, or Ca in feces on resin diet; Y is the percentage of ingested Na, K, or Ca in feces during the comparable control period (that is, either the preceding or following control period on the same basal diet); Z is the mEq. of Na, K, or Ca ingested on resin diet; a is the gm. cationic exchange resin ingested, that is,

$$\frac{(\text{net food intake}) \times (\text{per cent cationic exchange resin in diet})}{100}$$

Some difficulties arise in applying this method of calculation to the case of calcium, where the percentage unabsorbed in control experiments varies from 58 to 73. In *pair E*, for example, the unabsorbed calcium in the only control experiment is unusually high, and this leads to negative values when the effects of the resins are calculated. It is probably more reasonable to regard the latter values as zero.

Retention of minerals is rather high in some of the pairs, but it must be kept in mind that the animals were usually growing rapidly, and positive balances are to be expected. For example, Orent-Keiles and McCollum (13) observed that rats in their control group grew an average of 97.5 gm. in 15 weeks on a diet which contained⁸ 0.66 per cent Na, 1.16 per cent K, 1.14 per cent Ca, and 0.59 per cent Cl. During the 15 weeks, when correctly calculated (14), they found that these animals retained an average of 1156 mg. Na, 65 mg. K, 2351 mg. Ca, and 483 mg. Cl. From our knowledge of the composition of the mammalian organism, it would be more reasonable to expect a retention of about 150 mg. Na, 300 mg. K, and 200 mg. Cl for that amount of growth. Those of our balances which seem particularly high are of Na, K, and Cl in *pairs B* and *D* for the first 12 days. However, the amounts retained do not correspond to the composition of either the food or the urine, hence do not seem attributable to errors in determining food consumption, or to loss of urine. For the other periods and groups the balances are of about the expected magnitude.

There is some evidence that the basic resin Amberlite XE-58 bound chlorides in the intestine (*pair A*, period 2; hereafter referred to as 'Test A-2,' etc.) but the Ionac A-300 does not seem to have had more than a slight effect in this regard. In two tests (A-2 and C-2) the presence of the basic resin seems definitely to have increased the available capacity of the Amberlite XE-64, but in a third test (E-6) it had no effect. Any increase in capacity achieved in this way, however, was practically cancelled by the extra resin fed, i.e. it was no greater per total gram of resin fed.

⁸ Only the sodium content of their diet is specifically stated. The percentages of K, Ca, and Cl have been calculated (by the present authors) from their data for total intake of food and of these elements.

Results of the screening experiments have been summarized in table 3. The amounts of the major metallic elements bound, per gram of cationic exchange resin ingested, are given for each test. In general, the values are consistent with reasonable expectations: The total capacity utilized *in vivo* is about 20 to 25 per cent of the manufacturer's claim. In tabulating the amounts of the elements bound, the values used have ordinarily been those for the last 3 days of the period, rather than all 6. This has been done in order to eliminate the effect of metabolic lag. It will be noted from table 1 that the amounts bound during days 4 to 6 of a period are almost invariably higher than those for days 1 to 3, particularly when resin feeding followed a period on the basal diet. Values obtained for days 4 to 6 of a period probably represent, therefore, a more realistic appraisal of what might be expected in a long-term feeding experiment. Where the test followed a period on another resin regime, as was sometimes the case, an average value based on all 6 days has been used since no metabolic lag needs to be considered.

With respect to the idea of feeding a carboxylic-type resin partly in the potassium cycle,⁹ several points should be mentioned. To feed a resin in the hydrogen cycle evidently involves a loss of from 1 to 2 mEq. of fixed base per gram resin ingested.¹⁰ However, if the resin were fed partly in the potassium cycle and an exchange of potassium for sodium occurred, the net loss of alkali would be diminished, or perhaps eliminated. Data in table 1 indicate that such an exchange does occur. The amounts of potassium ingested per gram resin were as follows: Test no. C-6, 0.47 mEq.; test no. B-3, 0.93 mEq.; test no. D-6, 1.40 mEq. From the amounts of alkali lost per gram resin for these tests (see table 3), it can be seen that by comparison with tests B-2 and C-4, there was some saving of alkali reserve in all cases,— particularly in D-6. It can be seen further that to feed a resin partly in the potassium cycle decreases its combining capacity for both sodium and calcium, while increasing the urinary output of potassium. (NOTE: as will be seen in *pair 4*, table 2, a similar situation exists; less sodium and more potassium were bound by the resin, with an increased urinary output of potassium. Each gram of resin ingested contained 0.93 mEq. of potassium, and the alkali loss in the feces was about 1.25 mEq/gm., leaving a net loss of 0.32 mEq/gm. as against a loss of 0.90 mEq/gm., when the resin was fed in the hydrogen cycle; see *pair 3*, table 2. This is a somewhat more favorable comparison, relatively speaking, than found above.)

The experiment involving feeding of ion exchange resins in conjunction with a very low sodium diet produced a number of interesting results. The animals appeared quite healthy in every respect on these regimes with the exception that growth was subnormal in all pairs, and that one rat of the control pair died on the 17th day of causes unrelated to any dietary deficiency. This pair maintained small sodium and chloride balances, and adequate potassium balances. The calcium balances were very erratic, being unusually large for the second week, and unusually small for the other 2 weeks. Animals on the resin diets fared almost as well, in spite

⁹ The idea of feeding resins partly in the K cycle was suggested by Crismon in his presentation at Detroit (6).

¹⁰ Irwin *et al.* (3) feel that this acidosis is largely compensated by formation of ammonium salts in the kidney.

of the fact that in some pairs, for example, 2, 3, and 5, the presence of the resins in the diets had the effect of reducing the absorbed sodium and potassium to about 0.6 and 4 mEq/100 gm. of food, respectively. Feeding the resin partly as the potassium salt (*pair 4*) increased the potassium, and decreased the sodium, bound by

TABLE 3. IN VIVO BINDING POWER OF SOME CATIONIC EXCHANGE RESINS

TEST NO.	RESIN FED	CYCLE	PERCENTAGE IN DIET	MEQ BOUND/GM. CATIONIC EXCHANGE RESIN INGESTED ¹			
				Sodium	Potas- sium	Calcium	Total
<i>E</i> ² -5 ³	Win 3000	NH ₄ ⁺	5	0.64	0.33	(-0.32) ⁴	0.97
<i>A</i> -6	"	"	10	0.60	0.15	0	0.75
<i>D</i> -1	"	"	10	0.59	0.41	0	1.00
<i>D</i> -5	"	"	10	0.67	0.30	0	0.97
<i>D</i> -3	"	"	15	0.64	0.27	(-0.06) ⁴	0.91
<i>B</i> -2	Ambt. XE64	H ⁺	10	1.61	0.82	0.70	3.13
<i>C</i> -4	"	H ⁺	10	1.12	0.41	0.48	2.01
<i>C</i> -6	"	K ⁺	0.65	0.48	0.74	0.62	1.84
<i>B</i> -3	"	H ⁺	9.35	0.71	1.06	0.36	2.13
		K ⁺	1.30				
<i>D</i> -6	"	H ⁺	8.70	0.63	0.73	0.28	1.64
		K ⁺	1.95				
<i>C</i> -2	Ambt. XE64 & Ionac A-300	H ⁺	8.05	2.07	0.50	0.98	3.55
		OH ⁻	3.3				
<i>E</i> -6	Ambt. XE64 & Ionac A-300	H ⁺	6.7	0.87	0.53	0.42	1.82
		OH ⁻	3.3				
<i>A</i> -2	Ambt. XE64 & Ambt. XE58	H ⁺	6.0	1.46	0.68	0.63	2.77
		OH ⁻	4.0				
<i>E</i> -4	Ambt. XE66	H ⁺	5	0.67 ⁵	0.06	0.11	0.84
<i>A</i> -4	"	H ⁺	10	1.10 ⁵	0.54	0.70	2.34
<i>B</i> -5	Permutit XAC	H ⁺	10	0.33	0.18	0.50	1.01

¹ In arriving at these figures, values for the second half of the period have always been taken whenever the test immediately followed a period on the basal diet, in order to eliminate effect of metabolic lag in the first half. When the test followed a period on a resin regime, average value for the entire period was used (see text). ² *Pair No.*, table 1. ³ *Period No.*, table 1. ⁴ Since negative values are a reflection of the natural variability of calcium absorption in the control tests, they have not been subtracted in arriving at total capacity. ⁵ Resin contained 0.25 mEq. sodium/gm. when fed.

the resin. In *pair 6* these positions were exactly reversed.¹¹ In this experiment, *pair 5*, the addition of Ionac A-300 to the diet seems definitely to have increased the available capacity of Amberlite XE-64, but when allowance is made for the extra bulk

¹¹ A part of the sodium which appears to have been bound by this resin was contained in it when fed, and when correction is made for this amount (0.25 mEq/gm.) its sodium-binding power becomes virtually the same as that of the other resins.

of the basic resin, the effect is again practically cancelled. From the composition of the blood sera on the 21st day, there appeared to be no depletion of either sodium or potassium on any diet. The data on muscle indicate a slight depletion of potassium in all pairs, as compared to the values reported by other investigators (15, 16), but no depletion of sodium except possibly in *pair 2*. These results are somewhat at variance with those of Crismon (6) who found low sodium and potassium in the muscle of his animals although they were absorbing about 6.5 mEq. sodium and 11.5 mEq. potassium/100 gm. food, and the depletion period used in the present work was somewhat longer.

There is nothing in these results to suggest that any appreciable amounts of endogenous sodium and potassium were bound by the resins, although adequate binding capacities were available in most of the diets. If this mechanism plays any role in the relief of edema it must be a minor one, since it could only occur when the mineral content of the diet is so low that the sodium and potassium contained in the digestive fluids represent an appreciable fraction of the total anions available for binding in the intestine. The principal mechanism involved in the relief of edema would appear to be, therefore, a reduction of the amount of sodium absorbed from the food to the point at which the subject's diminished, but still definite, capacity to excrete sodium through the kidneys (17) becomes effective.

There is very little to choose among the various resins used in this 21-day experiment as to efficiency in binding anions. The individual resins were also remarkably constant in their effects from week to week. Sodium and potassium were bound in about the ratio of 1:6, except in cases where the picture was complicated by the presence of one of these elements in the resin itself. This ratio is practically the same as in the basal diet, 1:5.3. Calcium absorption was satisfactory in all of the pairs which received resin diets; the balances were most favorable in *pair 5*, followed by *pairs 2, 6, 3, and 4* in that order. Retention of calcium in the control pair was so erratic that no attempt has been made to calculate the amounts bound per gram resin in other pairs.

Data reported herein represent an extension, and a substantial summation, of many similar experiments conducted by the authors (18). These experiments adequately demonstrated the ability of several typical cationic exchange resins to bind sodium in the intestine, while at the same time permitting an adequate absorption of calcium.

SUMMARY

A number of ion exchange resins was tested for efficiency in binding cations *in vivo*. Dietary levels ranging from 5 to 15 per cent were tested, but ordinarily 10 per cent was used. All of the cationic exchange resins bound some sodium and potassium in the intestine; when fed in the hydrogen cycle they also bound some calcium, but this was not true of the resin which was fed in the ammonium cycle. About 20 to 25 per cent of the total capacity of the resin was usually utilized, with the ratio of binding of Na:K being nearly 2:1, although the dietary ratio was either 3:2 or 2:2. It was not definitely established here whether or not feeding a basic (anionic) exchange resin increased the available capacity of a resin of the carboxylic type. When

a resin of the latter type was fed partly in the potassium cycle, it bound sodium satisfactorily in the gut, but with less net loss of alkali to the animal than when it was fed entirely in the hydrogen cycle. At a 10 per cent dietary level the resins usually bound from 15 to 60 per cent of the sodium intake (after making due allowance for the amounts normally unabsorbed on the basal diet), and there was no outstanding difference among them beyond what could have been predicted from their rated capacities. The animals occasionally showed small negative balances of sodium, potassium and chloride on the resin regimes, particularly when their food consumption was low.

In a 3-week feeding experiment, other animals received a diet which was very low in sodium and chloride, but adequate in potassium and calcium. A control pair was able to maintain small sodium and chloride balances, along with large potassium and calcium balances. In the animals receiving the resin diets some sodium and very large amounts of potassium were lost in the feces. Nevertheless, they were able to maintain positive balances of both elements. Growth was not rapid, but there was no obvious evidence of the effects of sodium depletion, nor was any revealed by analysis of serum or muscle or autopsy. Muscle potassium was slightly low in all pairs, including the controls. Calcium balances were adequate in all of the pairs receiving the resin diets; there was no important difference among the various resins as to efficiency in binding sodium and potassium. It seems likely that the resins act to relieve edema almost entirely by binding exogenous sodium.

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CHANGES IN BLOOD OF THE RAT DURING ETHER AND BARBITURATE ANESTHESIA¹

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THE effect of anesthesia upon the chemical composition of the blood has not been adequately studied in the rat, although a few observations have been recorded. The following deals with the effects of ether, sodium amytal, sodium pentobarbital, and hexobarbital, and may prove helpful to those planning experiments involving the blood or plasma levels of glucose, albumin, globulin, non-protein nitrogen (NPN), chloride and cholesterol. It is of interest that a somewhat similar study was undertaken recently for the first time in the cat (1).

METHODS

Adult Sprague-Dawley males (150-350 gm.) were used in most of the experiments. The results were confirmed in another strain when it seemed desirable. Blood was taken by cardiac puncture (2), by use of a half-inch no. 23 needle and a syringe which had been rinsed with one per cent heparin (dissolved in 0.85% sodium chloride solution). From 2 to 6 ml. of blood were placed in a centrifuge tube, in which 0.1 mg. of heparin had been dried. The use of heparin maintained the normal electrolyte composition and avoided changes in corpuscular volume which might interfere with the analyses, as in the case of potassium oxalate (3, 4).

All analyses employed the Coleman junior spectrophotometer. Hemoglobin was determined as oxyhemoglobin, using a commercial hematin standard, according to Horecker (5), and checked for iron (6).

The tungstic acid filtrate of plasma was made by mixing 0.5 ml. of plasma, 3.0 ml. of water and 2.0 ml. of tungstic acid reagent. The latter was prepared each day by adding 2.5 parts of 10 per cent sodium tungstate to 17.5 parts of 0.147 N sulfuric acid. This adjusted the pH of the filtrate to 2.5, which we found optimal for protein precipitation, judged by obtaining a minimal value for the NPN in the subsequent Kjeldahl on the filtrate. The Kjeldahl digestion was done by the sulfuric acid-hydrogen-peroxide method, and ammonia determined by nesslerization (7).

Glucose was determined by the arsenomolybdate method of Nelson (8), read at 520 m μ , using the tungstic acid filtrate. The values were found to be 5 to 10 mg. per cent higher than those obtained with zinc hydroxide. It should be noted that the plasma level of glucose is considerably higher than that for whole blood.

Chloride was determined by the Van Slyke and Hiller (9) modification of the Sendroy method. We employed the colorimetric modification suggested by the Aloe Technical Manual in which the 2-ml. aliquot, titrated by Van Slyke and Hiller, is made up to 25 ml. with distilled water plus 200 mg. of sodium iodide, and read in the photometer at 475 m μ . To check on the possible importance of the chloride shift resulting from a loss of carbon dioxide, blood was drawn, handled as usual and, in addition, shaken in the test tube. A sample was then centrifuged and the plasma (pH 7.8-8.1) analyzed

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for chloride. The rest of the blood was equilibrated with 5 per cent carbon dioxide in oxygen, and the plasma (pH 7.3-7.4) centrifuged off under oil. The mean and standard deviation for 5 different plasmas so tested was 606 ± 8 mg. per cent (as NaCl) before equilibration, and 598 ± 13 mg. per cent after equilibration. These differences did not warrant the taking of special precautions to prevent the loss of carbon dioxide.

Proteins were determined by Weichselbaum's (10) modification of the biuret test. The method was standardized against the Kjeldahl. For the precipitation of globulin we employed sodium sulfite, as suggested by Campbell and Hanna (11), who found the reagent to act independently of temperature. To be sure that this would apply to the rat, we carried out precipitations at 22° and 37° C. and covered the range of concentrations from 10 to 28 per cent of sodium sulfite. The curve relating precipitation to final salt concentration showed a discontinuity in the form of a small plateau between 19 and 22 per cent sodium sulfite. We therefore selected 19.5 per cent as our standard working concentration. The curves obtained at 22° and 37° were identical.

Total cholesterol was done by Kaye's method (12). Our values for the Sprague-Dawley and Osborne-Mendel rats were considerably higher than those in the literature: Wistar and Long-Evans strains 48 (25-73) mg. per cent (13); Wistar strain 61 ± 2.6 mg. per cent (14). Since our tests on 5 normal men gave values in the usual range (179, 196, 190, 214 and 218 mg. per cent) this difference is attributed to the strains employed. This conclusion is strengthened by the fact that another strain in our colony, the Tumblebrook hooded, averages around 70 mg. per cent.

EXPERIMENTAL

It is convenient to start with the results obtained with ether. Anesthesia was induced by placing the rat in a closed 2-l. jar, on the floor of which lay a wad of ether-soaked cotton, covered by a coarse wire mesh. Sufficient anesthesia for cardiac puncture occurred in less than one minute in the Osborne-Mendel rat, and in 1 to 1.5 minutes in the Sprague-Dawley. The animal was then removed from the jar, placed on its back, and cardiac puncture performed during the next minute or two. To prolong anesthesia after removal from the ether jar, a 25-ml. beaker containing a wad of ether-soaked cotton was placed over the animal's snout.

Table 1 combines the data of 4 experiments made on 4 different days which illustrate the effect of duration of ether anesthesia upon the chemical composition of the blood. Two experiments were made with Osborne-Mendel males, one with Sprague-Dawley males, and one with Sprague-Dawley females. The rats were anesthetized for varying periods of time and a single cardiac puncture performed, after which the animal was discarded. The analyses are grouped for 3 periods, 0-5, 5-10, and 10-20 minutes. The table shows that ether anesthesia during the first 20 minutes affects only the concentration of glucose. This is equally true for animals allowed free access to food, or those starved for 24 hours. In addition, the table shows that 24 hours of starvation lowers the hemoglobin, total protein, NPN and glucose. This finding has been consistently true in the 4 strains of rats we have employed and is described more fully later (15).

The plasma glucose concentration during the course of ether anesthesia is plotted in figure 1, where a smooth curve has been drawn through the points. Where does this curve intercept the Y-axis at zero time? The points taken under anesthesia did not extend below 1.5 minutes because it required about one minute for anesthesia to occur and another half-minute to draw the blood, working as quickly as possible. The 6 points plotted between 0.5 and 1.5 minutes (solid black) were based on animals stunned by a blow on the head; the stopwatch having been started immediately

the animal was grasped. They indicate that the slope of the curve was no greater, and probably was less, during the first 1.5 minutes than it was subsequently. If we assume that the average for the 6 stunned animals in fact represents the value at zero time it would be 132 ± 2 mg. per cent. If we extrapolate the curve through them to zero, the minimum value would be 128 mg. per cent. Normally, blood would be drawn in the interval 1.5 to 3 minutes after exposure to ether, where the plasma sugar ranged from 136 to 146 mg. per cent, or about 10 mg. per cent higher than the true, unanesthetized value. That these data are of some general significance is shown by the fact that during the past 8 months in other experiments we have determined the plasma sugar concentration, using the same procedure, on 71 Sprague-Dawley and Osborne-Mendel rats. For the pooled data, the mean and standard deviation were 141 ± 10 mg. per cent.

To determine whether or not the sugar concentration of blood drawn from the heart would agree with that obtained from the tail, 4 rats were subjected to both

TABLE 1. ANALYSES DURING ETHER ANESTHESIA¹

STARVED ²	PERIOD	HEMOGLOBIN	TOTAL PROTEIN	A/G	CHLORIDE ³	NPN	GLUCOSE
	<i>minutes</i>	<i>gm. %</i>	<i>gm. %</i>		<i>mg. %</i>	<i>mg. %</i>	<i>mg. %</i>
Yes	0-5	15.6 (16)	6.9 (9)	1.99 (5)	607 (11)	32.2 (4)	141 (16)
	5-10	15.7 (7)	6.8 (2)	1.96 (1)	608 (6)	31.8 (1)	160 (8)
	10-20	15.5 (9)	6.8 (5)	2.01 (4)	610 (8)	32.9 (4)	217 (9)
No	3	16.7 (2)	7.2 (2)	2.00 (2)	611 (2)	41.3 (2)	184 (2)
	7	16.4 (2)	7.3 (2)	2.04 (2)	607 (2)	40.9 (2)	251 (2)
	16	16.5 (2)	7.1 (2)	2.00 (2)	600 (2)	41.2 (2)	292 (2)

¹ A single sample of blood was taken from each animal, but a complete set of analyses was not always performed. Except for hemoglobin, the concentrations refer to plasma. The bracketed figures refer to number of animals in each average. ² For 24 hours. ³ As NaCl.

cardiac and tail vein puncture. The tail was placed in water at 46° C. for 20 seconds before blood was drawn. The results for whole blood analysis at 2.5, 3, 4 and 5 minutes after exposure to ether were, respectively, for heart blood, 97, 101, 104 and 115 mg. per cent; and for tail vein blood, 97, 101, 102 and 126 mg. per cent.

Ether anesthesia in the dog and in the cat induces a rise in hematocrit and plasma proteins; and in the dog a decrease in plasma and interstitial fluid volume has been demonstrated (16, 1). Such findings contrast sharply with the data of table 1. On the other hand, in 16 patients under ether anesthesia without parenteral fluids for 1 to 3 hours, Stewart and Rourke (17) found no significant change in the hematocrit, plasma protein or serum chloride. The NPN was rather variable, and the mean change for the group was plus one per cent. It thus appears that the rat resembles man rather than the dog or cat in its reaction to ether. Stewart and Rourke (17) also found that the blood and plasma volumes fell 14 per cent, and the interstitial fluid volume rose 27 per cent. They commented upon the fallacy of interpreting changes in blood concentration directly in terms of blood volume.

Quimby and Saxon (18) concluded that hemodilution occurred during ether anesthesia in the rat because the specific gravity fell from 1.059 to 1.054 and the red cell count fell from about 8 to 7 million. Their data are in contrast to ours, and also disagree with the conclusions of Crafts (19) who found no change in either the red or white cell count of rat blood obtained by cardiac puncture regardless of the depth of ether anesthesia.

Ether anesthesia was without effect upon the plasma level of total cholesterol, in both starved and fed animals. For the Sprague-Dawley strain, the mean and standard deviation for 10 rats tested at 2 to 7 minutes was 114 ± 12 mg. per cent, and for 9 rats at $7\frac{1}{2}$ to 18 minutes 111 ± 10 mg. per cent.

The barbiturates were given intraperitoneally as freshly prepared 5 or 10 per cent solutions of the sodium salts dissolved in water. In the case of the Sprague-Dawley

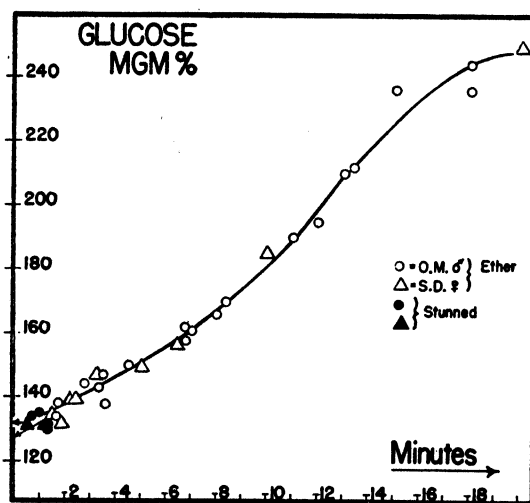


Fig. 1. PLASMA GLUCOSE following exposure to ether. The solid symbols plotted during the first 2 minutes are for animals stunned by a blow on the head. The animals had fasted for 24 hours.

rat, 50 mg/kg. of sodium pentobarbital produced deep anesthesia in 5 to 10 minutes, which lasted for about 45 minutes. The Osborne-Mendel rat required only 60 per cent of this dose. Sodium amytal was not as suitable for the production of deep anesthesia, but at 65 mg/kg. produced a light anesthesia in 5 minutes quite suitable for cardiac puncture, and from which partial recovery was noted 25 minutes after injection. Sodium hexobarbital (evipal) at 100 mg/kg. induced anesthesia, which lasted for about 20 minutes, in less than 4 minutes in the Sprague-Dawley rat. In the Osborne-Mendel, the effect lasted for about one hour.

Because of the interval between the injection of the barbiturate and the occurrence of sufficient anesthesia to permit cardiac puncture without restraint, the curves in figures 2 and 3 begin at 6 minutes for amytal and at 10 minutes for pentobarbital. No effect of anesthesia was noted upon the chlorides or the A/G ratio, and only a trivial fall occurred in the NPN; these have been omitted from the figures.

The results of 4 separate experiments for amytal and pentobarbital have been

combined in figures 2 and 3. In each experiment a group of rats was injected at zero time, and at various intervals thereafter a pair was subjected to cardiac puncture. No animal was used twice. Each point on the graph, therefore, represents the average of 2 animals, and the various curves are based on a total of 14 to 20 animals. For comparison, one pair of animals in each experimental group was subjected to ether instead of barbiturate anesthesia, blood being drawn 1.5 to 2 minutes after exposure to ether had begun. These data also are listed in each figure. The animals were allowed free access to food up to the time of the experiment, and the blood levels therefore correspond with those in the bottom half of table 1. The data for the etherized animals agreed quite well with those in table 1 except in the case of hemoglobin, which was about one gram per cent higher. All of our subsequent hemoglobin determinations have agreed with the data of table 1, and we therefore feel that the standard employed

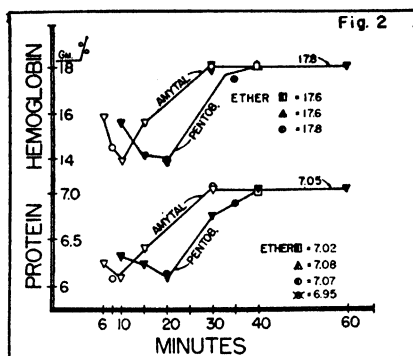


Fig. 2. HEMOGLOBIN AND TOTAL PROTEIN LEVELS following intraperitoneal injection of barbiturate. Values obtained after 2 minutes exposure to ether are recorded for comparison. The animals had free access to food.

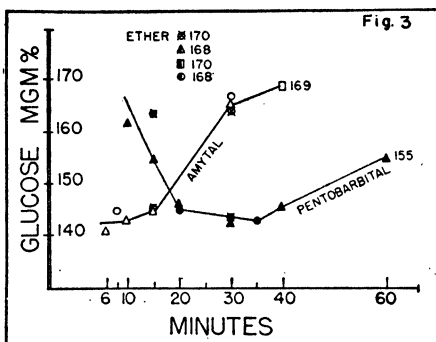


Fig. 3. PLASMA GLUCOSE CONCENTRATION following intraperitoneal injection of barbiturate. Values obtained after 2 minutes exposure to ether are recorded for comparison. The animals had free access to food.

during the interval covered by figures 2 and 3 may have been off by about 6 per cent. This, however, does not affect any argument concerning changes in hemoglobin concentration during the course of a particular experiment.

The shape of the curves in figures 2 and 3 suggested that when anesthesia occurred, the levels of hemoglobin, protein, and glucose had fallen from their initial normal values and that, as anesthesia lessened during recovery, a rise to the normal value ensued. Both drugs produced the same changes, though amytal acted more quickly and for a shorter time. Five of the 6 curves showed a terminal plateau which had its origin before recovery from anesthesia was complete. In the case of hemoglobin and protein, the plateau levels agreed well with those obtained under ether, and may therefore be considered normal.

The curves for hemoglobin and total protein paralleled one another closely, as if under the control of a single mechanism. Since the chloride and NPN were constant, a possible explanation would be the movement of interstitial fluid into the vascular

compartment. The literature indicates, however, that changes during barbiturate anesthesia are not altogether understood, and are complicated by species differences. Thus, pentobarbital causes a fall in hematocrit, plasma proteins and white cell count in the cat and dog (1, 20). In the dog, splenectomy will partially reduce the fall in erythrocyte count (21), but not in the rabbit or rat (22). In the dog, the blood volume remains constant while the plasma volume rises (16).

To interpret the curves for glucose in figure 3, it should be recalled that exposure to ether for about 2 minutes will raise the plasma glucose level about 10 mg. per cent above the normal value. In figure 3 the curve for the amytal series rises to 165 mg. per cent at 30 minutes, and 169 per cent at 40 minutes. These are in close agreement with the 169 mg. per cent average obtained under ether anesthesia (1.5-2 minutes exposure) on the same days that the amytal tests were executed. Likewise, the first points on the pentobarbital curve are in the neighborhood of 160 mg. per cent. It would appear that both amytal and pentobarbital depress the plasma sugar level, which returns to normal as the effect of the anesthetic wears off.

The data for hexobarbital, the most rapidly acting barbiturate of those tested, were consistent with the foregoing, and allowed the early changes to be demonstrated more readily. Experiments were done on 3 different days during the course of a month, employing both males and females of the Osborne-Mendel and Sprague-Dawley strains. No differences were apparent and all of the data were plotted together in figure 4, where each point represents analysis of blood from a single rat, no animal having been used more than once, and each curve being based on 14 to 17 animals. The chlorides were unchanged and have not been plotted: the mean and standard deviation for 8 animals tested in the interval 3.5 to 16 minutes was 607 ± 2 mg. per cent (as NaCl). The effect of the barbiturate was unchanged by starving the animals for 24 hours prior to testing, although the usual fall in hemoglobin, protein and glucose due to starvation occurred.

As indicated by the symbols in figure 4, three different doses of sodium hexobarbital were used, 75, 90, and 100 mg/kg. Anesthesia sufficient for cardiac puncture occurred in about 3 minutes, but at the lowest dose did not occur in all of the animals so treated, and when it did occur was of shorter duration (by 30-50%). Nevertheless, the figures show that during the interval studied, the chemical changes in the blood were similar at all 3 dose levels.

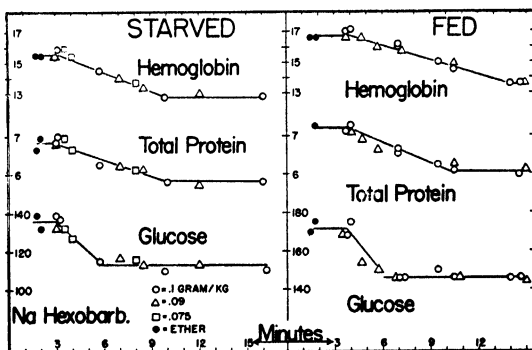
The curves in figure 4 appear to be bi- or triphasic. Until about the fourth minute the values for hemoglobin, protein and glucose agreed with those found under brief ether anesthesia. In the case of glucose, an abrupt drop of about 25 mg. per cent occurred during the fourth to sixth minutes, after which the concentration was constant. Likewise, values for hemoglobin and protein before the fourth minute were equal to those under brief ether anesthesia. Thereafter they fell, tending to parallel one another, from the fourth to about the tenth minute. Comparing these results with those for amytal and pentobarbital, it is seen that a similar fall in hemoglobin and protein occurred, the mechanism of which might be the movement of interstitial fluid into the vascular compartment. Also, a fall in glucose occurred in all cases, evidently due to a decline from the normal level.

DISCUSSION

It is evident that ether and the barbiturates have quite different effects upon the chemical composition of the blood of the rat, as might have been predicted from work on other species (16, 23-25). What is especially striking in the rat is the rapidity with which the changes occur, both in the starved and fed animal. Since the state of feeding also affects the hemoglobin, NPN, protein and sugar levels, a great variety of results can be obtained unless standardized conditions are employed. Thus an animal tested under hexobarbital after a 24-hour fast would have a hemoglobin of 13 gm., a total protein of 6 gm., and a glucose of 115 mg. per cent. The same animal fed and tested under ether would have a hemoglobin of 16.5 gm., a total protein of 7.2 gm., and a glucose of 170 mg. per cent.

The choice of standardized conditions for bleeding experimental animals introduces an arbitrary element into the experimental design. No doubt the first requirement for any procedure is that it shall give consistent results. Other goals are minimal

Fig. 4. HEMOGLOBIN, TOTAL PROTEIN, AND PLASMA GLUCOSE LEVELS following intraperitoneal injection of sodium hexobarbital. The solid circles during the first 2 minutes are for animals exposed to ether, for comparison.



derangement of the systems under examination and simplicity. Whether the animals should be starved or fed will depend upon the special requirements of the experiment in question, and, as far as the present results go, will not affect the choice of anesthetic. From the standpoints of derangement and of simplicity, however, it would appear that ether is the anesthetic of choice. It affects only the glucose, and because of the rapidity with which it acts, this derangement can be minimized. It has the further advantage that recovery is a matter of a few minutes, that it is cheap, and that it is easy to administer.

SUMMARY

Chemical changes in the blood of the rat during ether and barbiturate anesthesia were studied. The barbiturates depressed the levels of hemoglobin, total protein, and glucose. The speed and magnitude of the changes varied with the drug employed (amytal, pentobarbital and hexobarbital), which imposed a limitation upon their value for blood sampling. Ether elevated the blood sugar, but this effect was only

about 10 mg. per cent during the first several minutes of anesthesia. Ether did not change the hemoglobin, total protein, A/G ratio, NPN, chloride or cholesterol.

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EMOTIONAL HYPOTHERMIA IN RABBITS¹

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IT HAS been known for 140 years or more that rabbits become hypothermic when subjected to restraint in various ways. Ito (1) reviews the extensive early literature on this point. The hypothermia has usually been ascribed to diminished heat production following immobilization, and to increased heat loss due to exposure of greater skin areas and especially of the thin-furred belly. Ware, Hill and Schultz (2) found that tight binding with adhesive tape induces anoxia and hypothermia. Sinelnikoff (3) observed persistent hypothermia in rabbits handled at frequent intervals for determination of rectal temperature: he attributed the hypothermia to fear and states that 'training' of the rabbits may abolish the effect. The mechanism was not discussed.

METHODS AND RESULTS

In the early stages of a study of pyrogen fever in rabbits (4) light restraint was imposed so that rectal and ear temperatures might be recorded continuously. The restraining cage used was a tunnel-like structure made of one half inch mesh wire cloth and attached to a wooden floor. The rabbits were held in normal sitting posture without extension and without compression. This procedure almost invariably induced hypothermia, rectal temperature falling as much as 2.7°C. in 30 to 90 minutes. Occasionally, a stable temperature was established at the depressed level. Usually, the process was reversed and a stable temperature was established at or near the control level several hours later. Sometimes a hyperthermic phase followed the hypothermic. The response to pyrogen injections during hypothermia was abnormal and was often so even after a stable temperature had been reestablished at the control level. Even moving the animal to a strange cage or keeping it under scrutiny on a table might induce some hypothermia. The phenomenon could usually be avoided entirely by periodic use of a clinical thermometer for rectal temperature determinations, or by fixing a short thermocouple in place and attaching it temporarily to potentiometer leads for determinations, leaving the animal free and undisturbed at other times, preferably in its living quarters.

Figure 1 *I* shows the course of hypothermia in an animal restrained at an environmental temperature of 25°C. The initial temperature and respiratory rate determinations were taken while the animal was still in its living quarters. Immediately following restraint (which was carried out without exciting the animal and in itself caused little change in rectal temperature) the ears became greatly flushed

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and their temperature (recorded by a small thermocouple previously inserted into a subcutaneous pocket on the dorsum of the ear) rose to the level of rectal temperature. At the same time, fast polypnoea was established. These heat dissipation mechanisms remained active for $1\frac{1}{2}$ hours, during which time rectal temperature fell 2.5°C . Constriction of the ear vessels and marked respiratory slowing then resulted in a slow

Fig. 1 I. EFFECT of restraint on rectal temperature, ear temperature and respiratory rate per minute.

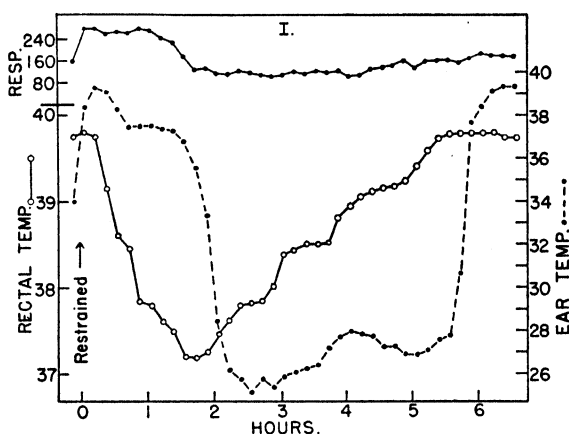
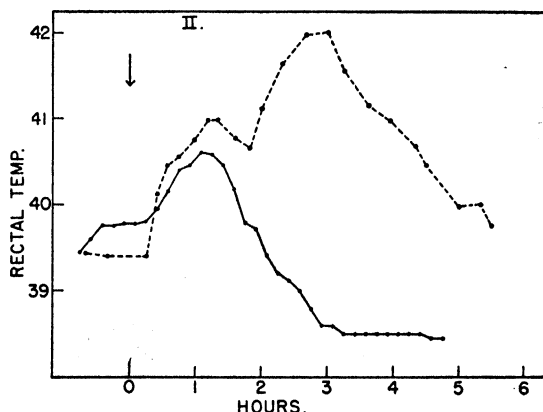


Fig. 1 II. EFFECT of pyrogen injection on a free animal (broken line) and on a restrained animal following recovery from hypothermia.



return of rectal temperature to normal $5\frac{1}{2}$ hours after restraint, when the ears became hot once more, but fast polypnoea did not return. In this case rectal temperature again declined, but more slowly, and a stable temperature was established about 1° below normal 8 hours after restraint.

In 51 experiments on 36 animals carried out at environmental temperatures from 18° to 30°C , restraint caused activation of heat loss mechanisms and some hypothermia in 50 cases. In 46 of these (33 animals) hypothermia exceeded 0.5°

(mean 1.18 ± 0.088 ; range 0.0 to 2.7°). At environmental temperatures above 30° heat loss mechanisms are strongly active in the free animal and restraint causes little or no hypothermia.

Oxygen consumption measurements under similar conditions of restraint show that heat production is not significantly reduced. Despite the pronounced hypothermia, shivering does not occur during the falling phase and is not often seen during recovery, unless the animal is exposed to cold.

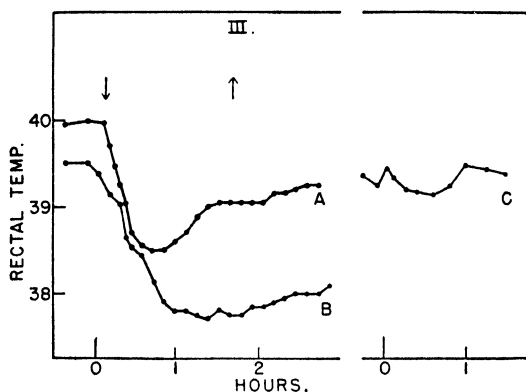


Fig. I III. EFFECT of cold exposure on development of hypothermia.

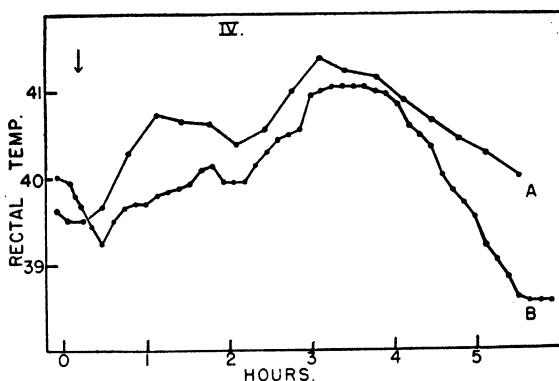


Fig. I IV. EFFECT of pyrogen injection immediately following restraint. For details, see text.

In figure I II the effect of intravenous injection of a pyrogen (5 gamma of 'Pyromen', a standardized preparation of pyrogen from *Pseudomonas aeruginosa*, generously supplied by Dr. N. M. Nessett of the Baxter Company) on a restrained animal is compared with the usual effect of the same material on an unrestrained animal. The animal was restrained 3 hours before injection and developed 1.8° of hypothermia, from which it had recovered completely. Beginning about an hour after injection, persistent ear vasodilation and mild polypnoea interrupted the fever and quickly lowered the rectal temperature until 1.25° of hypothermia had developed. Temperature then stabilized at this level. In the unrestrained animal similar interruption of the temperature rise occurred 75 minutes after injection (this is usual) but secondary

inactivation of heat loss mechanisms carried the animal to a high fever lasting 6 hours with no subsequent hypothermia.

Figure 1 *III*, *graph C*, shows the effect of restraint (carried out at zero time) on rectal temperature in an animal that had been exposed to 4°C. for an hour and was kept at that temperature after restraint. The animal had established ear vasoconstriction and a slow respiratory rate very soon after being placed in the cold and with negligible fall in rectal temperature. Restraint did not disturb the efficiency of thermoregulation appreciably. *Graphs A* and *B* in this same figure show the effects on the same animal of restraint at 27° (*B*) and of restraint at 27° followed by exposure to 4° when activity of the heat loss mechanisms had been established (*A*). In *graph B*, respiratory slowing began after 70 minutes and ear vasoconstriction after 90 minutes of restraint. Shivering did not occur and the rise in rectal temperature was very slow. In *graph A* the animal was placed in the 4° box 6 minutes after restraint (first arrow). Respiratory slowing and ear vasoconstriction became perceptible simultaneously 15 minutes later and were maximal by 24 minutes. Shivering began after 42 minutes exposure and the rise in rectal temperature was relatively rapid, but on returning the animal to 27° (second arrow) shivering ceased and the temperature rise became more gradual.

Thus prior exposure to cold inhibits the phenomenon almost entirely and exposure to cold after activity of the heat dissipation mechanisms has been established cuts short the paradoxical cooling process but not with sufficient rapidity to prevent a sharp fall in body temperature.

When a pyrogen is injected in the early stages of emotional cooling, activity of the heat dissipation mechanisms is inhibited as in the free animal and a more or less typical fever develops. Figure 1 *IV* shows the response of the same animal represented in 1 *III* to pyrogen injection (time marked by the arrow) when unrestrained (*A*) and when restrained 12 minutes before injection (*B*). The cooling process started by restraint continued unchanged for 16 minutes after injection (cf. fig. 1 *III*, *B*) when striking respiratory slowing and ear vasoconstriction took place with consequent rise in temperature. Complete inactivation of heat loss mechanisms took 20 minutes, as in the free animal. The course of the resulting fever resembled that in the free animal but the small magnitude of the first rising phase and the marked hypothermia that developed following fever are atypical.

DISCUSSION

The rabbit is notorious for inefficiency of thermoregulation but the degree of hypothermia often developed under restraint is far outside the range of normal variations in free animals. The hypothermia is due to strong activation of the normal mechanism of heat dissipation and it seems certain that it is mediated through the thermoregulatory centers since shivering is inhibited. Probably the hypothermia due to handling described by Sinelnikoff (3) and others is a mild form of the phenomenon here described for although he states that hypothermia is accompanied by constriction of the ear vessels and failure of polypnoea, this probably applies to the steady state of depressed temperature. Most of the hypothermia resulting from such procedures as tying an unanesthetized rabbit to an animal board in an extended position

is probably due also to activation of heat loss mechanisms rather than to inadequate calorogenic compensation for increased exposure of surface, for even shorn rabbits can tolerate prolonged exposure to environmental temperatures below 0°C. with only slight depression of temperature (4).

The hypothermia cannot be due to compression or anoxia since it is fully reversible without change in conditions, does not occur in animals defending against cold before restraint, and may occur in a totally unrestrained rabbit placed in unusual surroundings. It is not a secondary result of the handling incidental to inserting a thermocouple etc., for an animal that has gone through these procedures usually shows no significant change if the restraining cage is left off, but promptly starts lowering its temperature when the cage is placed over it gently.

It seems legitimate to describe this phenomenon as 'emotional' hypothermia, probably dependent, as Sinelnikoff suggested, on modification of cerebral cortical control over thermoregulatory centers. Hormonal or other indirect links may be involved, but, whatever the details, it seems clear that the direct thermal sensitivity of the thermoregulatory centers (supposedly the main mechanism of temperature regulation in rabbits) can be overridden by factors arising out of an emotional state. The fact that exposure to cold tends to inhibit the phenomenon even when already started suggests that cutaneous sensations are a more important factor in the defense of rabbits against cold than is commonly supposed, as does the fact that shivering accompanies recovery from hypothermia in animals exposed to cold but is rarely seen in those exposed to moderate temperatures, although the latter are more hypothermic. The fact that pyrogen injections inhibit emotional cooling could be interpreted in several ways. It is consistent with the view recently expressed (4) that pyrogens act, not on the primary thermoregulatory centers, but on the motor mechanism of thermoregulation at lower levels in the brain stem.

These observations have important bearing on the practical conduct of thermoregulation experiments on rabbits, and on methods of testing solutions for pyrogen contamination. Molitor *et al.* (5) have suggested that rabbits used for pyrogen testing should be confined in boxes or stalls after insertion of a rectal thermocouple, and 60 to 90 minutes allowed for stabilization of temperature before the injection is made. The statement that in this period the rectal temperature falls an average of 0.5°C. from the value recorded immediately after removal of the animals from their living quarters suggests that this treatment also induces hypothermia. The mean control temperature at this time (39.03°) is lower than that usually observed in free rabbits (6) and the minimum control value given (37.1°) represents decided hypothermia. From figure 1 I it is evident that animals injected with a totally inert solution 60 to 90 minutes after restraint might show a spontaneous rise in temperature far in excess of the 0.6° suggested by McClosky *et al.* (7) as a criterion of pyrogenic activity. On the other hand restrained rabbits may fail to show a 'significant' response to a potent pyrogen (fig. 1 II). It is desirable, therefore, that rabbits used for pyrogen testing, and for studies on thermoregulation generally, should not be restrained, and preferably should be left in their normal living quarters. The results when pyrogen is injected immediately after restraint suggest that this procedure might be satisfactory but no tests have been made with weak doses of pyrogen. It is probable that with

frequent use many rabbits could be trained to tolerate mild restraint without disturbance of thermoregulation. But since rabbits readily become refractory to pyrogens they cannot properly be used for pyrogen testing more often than once every 3 weeks. Animals used at irregular intervals over a period of several months showed no indication of becoming used to restraint, nor did animals restrained daily for 7 days

SUMMARY

Light restraint of rabbits in normal posture and without compression may induce profound hypothermia by paradoxical activation of heat loss mechanisms. The effect is usually reversed but only after several hours. It is largely inhibited by exposure to cold, and completely inhibited by the injection of pyrogens, but the response to pyrogens may be abnormal both during the hypothermic phase and after recovery. The phenomenon is attributed to emotional factors.

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DO KANGAROO RATS THRIVE WHEN DRINKING SEA WATER?¹

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CONSIDERABLE attention has been given to the question of whether or not sea water could be utilized by mammals for drinking. Practical attempts to use sea water for man have been settled in disfavor of such use. The excretory system cannot handle the high salt concentrations, and some treatment of the water is necessary to remove the salts completely or partly.

Other mammals, however, with a higher excretory ability might be able to utilize sea water if no supply of fresh water were available. The question has been raised for seals and whales, which certainly do not have access to fresh water. The work of Irving (1) showed that seals living on fish apparently get enough water for their needs (urine formation and evaporation from the respiratory tract) from the water content of their food and from oxidation of the hydrogen in the foodstuffs. Similar calculations were made by Krogh (2) to show that whales also, even when living on crustacea with higher salt content than fish, apparently do not necessarily have to resort to drinking sea water. Whether or not whales or seals habitually or occasionally do drink sea water cannot easily be determined.

The question of whether or not land mammals can substitute sea water for fresh water was experimentally attacked by Adolph (3). The main problem is whether or not the urine can be excreted with salt concentrations sufficiently higher than the ingested that water remains available for the body. For his experiments Adolph used white rats, which have a more powerful excretory system than other laboratory animals, but access to sea water proved to have almost the same effect as complete deprivation of water.

As previously reported (4) the rodent *Dipodomys merriami* (kangaroo rat) has a very effective excretory system with a maximum electrolyte concentration in the urine (1200 mN) more than twice as high as sea water (580 mN). In their natural desert habitat these animals have free water only during occasional rains and at rare occasions of dew formation (5), and certainly never find ocean water. The high excretory ability must be considered an adaptation to the highest possible economy in their water balance, and they actually maintain water balance without drinking, on a diet of dry grain only (6).

Drinking of Sea Water. It seems quite reasonable to expect that kangaroo rats might have the ability to utilize sea water for drinking, provided the high content of magnesium (52 mEq.) and sulfates (28 mEq.) can be tolerated. The major difficulty is to make the kangaroo rats drink, because normally they do not need drinking water, and it would prove too optimistic to expect them to be more enthusiastic when offered sea water.

However, if kangaroo rats are offered dry food with particularly high protein

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content (soy beans) they cannot excrete the large amounts of urea formed, and hence they get into negative water balance unless additional water is given (7). It appeared that under these circumstances kangaroo rats will accept sea water as well as fresh water for drinking.

From the graph in figure 1 it appears that 4 animals kept on dry soy beans alone lost weight rapidly, while 3 given fresh water or 5 given sea water for drinking maintained their weight after an initial drop, and eventually even gained in weight. The initial drop was somewhat greater in the sea water group, but afterwards they gained at the same rate as the fresh water group and also ended up at the same level after 19 days.

In this experiment the sea water was from the Atlantic coast of New Jersey. The concentration of electrolytes, measured as electrolytic conductivity, was equal to 545 mN NaCl. Later 2 additional experiments were made, each with 4 animals in each group. In these cases Pacific ocean water was used, the electrolyte concentration

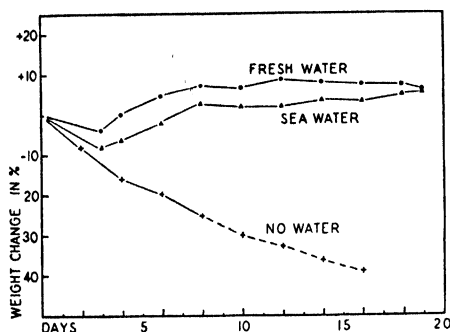


Fig. 1. WEIGHT CHANGES IN ADULT KANGAROO RATS kept for 19 days on a diet of soy beans, given fresh water, sea water, or no water for drinking. The weight changes are given in percentage of the initial weight, which was around 34-40 gm.

being 585 mN. Also in these experiments the sea water groups maintained weight as well as the fresh water groups.

Plasma Concentrations. It is of considerable interest to determine whether or not kangaroo rats maintain normal water and salt balance when consuming sea water and if they are able also to excrete the large amount of urea simultaneously imposed by the diet. Table 1 shows that the plasma electrolyte concentrations, after 19 days, are the same in animals drinking sea water and fresh water. Therefore, the animals apparently have the capacity of excreting completely the excess of salts ingested with the sea water.

The plasma urea concentrations are all within the normal range for kangaroo rats maintained on a grain diet (6), so that the drinking water, whether it is fresh or sea water, is sufficient for excretion of the large quantities of urea.

Urine Concentrations. Under these conditions we expected to find extremely high urine concentrations, particularly of electrolytes, in the animals drinking sea water. Samples were taken from the bladder when the animals were killed and analyzed immediately. Table 2 shows that 2 kangaroo rats had urine electrolyte concentrations far above that of the ingested sea water, and close to the concentration limit for their kidneys. However, 3 of the sea water animals showed urine concentrations

much below that of the sea water. Since all animals were observed to drink (variations from 1 to 5 ml. daily) and since salts were not retained in the body, the explanation might be that the animals periodically excrete very concentrated urine, and that in other periods the excretory system therefore need not work close to maximum performance.

Results of later work on the kidney clearance for various substances showed that the response to a single dose of 700 mN NaCl solution is a water diuresis, and not until several hours later do urine concentrations increase. Since the kangaroo rats

TABLE 1. PLASMA CONCENTRATIONS OF ELECTROLYTES AND UREA IN KANGAROO RATS AFTER 19 DAYS OF DRINKING SEA WATER, OR FRESH WATER, RESPECTIVELY

DRINKING SEA WATER		DRINKING FRESH WATER	
Electrolytes	Urea	Electrolytes	Urea
mN	mM	mN	mM
167	13.8	157	11.9
156	13.1	156	13.6
155	12.1	155	11.9
155	11.8		
153	10.6		
Av.: 157	12.3	156	12.5

TABLE 2. URINE CONCENTRATIONS OF ELECTROLYTES AND UREA IN KANGAROO RATS AFTER 19 DAYS OF DRINKING SEA WATER OR FRESH WATER, RESPECTIVELY

DRINKING SEA WATER		DRINKING FRESH WATER	
Electrolytes	Urea	Electrolytes	Urea
mN	mM	mN	mM
1040	2010	397	1290
975	2600	395	1547
470	1495	298	851
393	645		
150	423		
Av.: 606	1435	363	1229

which were offered salt water drank only during part of the night and did not touch water during the day, the ingestion of sea water came as a brief load, and it could not be expected that all the animals would show the very high urine concentrations simultaneously.

This physiological phenomenon has some bearing on the observation made by Fetcher (8) that the figures for urine concentrations in whales often are far below what would be expected from the salt content of the food. Fetcher rightly says that it would be impossible for whales to afford such comparatively dilute urines except after excessive renal work. There was no evidence in that direction, and many suggestions have been made of mechanisms by which whales could clear the

large amounts of salts which apparently did not appear in the urine. However, high urine concentrations (up to 820 mN Cl) are occasionally found in whales (2), and their mechanism for eliminating excess of salt might very well work along the same pattern as in kangaroo rats, i.e. that the salts are excreted in the highest concentrations in periods only, and the excretory system in other periods works at a moderate level.

Further work on the renal function in kangaroo rats is in progress.

SUMMARY

The desert rodent *Dipodomys* (kangaroo rat) is able to utilize sea water for drinking. It can excrete the large amounts of electrolytes and maintain normal water balance, also under simultaneous excess load of urea.

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WATER AND ELECTROLYTE DISTRIBUTION IN BLOOD AND TISSUES IN SPLENECTOMIZED DOGS BEFORE AND AFTER HYPOTONIC SALINE INJECTIONS¹

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IN THE preceding paper (1) the quantitative changes in the water and electrolyte distribution in blood and tissues in control dogs with spleens, following the injection of hypotonic saline solutions, were submitted. Data on control dogs showed that after infusions of large volumes of 77 mM NaCl, the cell volume and the red cell blood counts were not decreased in the control animals even though a dilution of the plasma occurred as denoted by the decreased concentrations for serum proteins and increased concentrations for serum water. It seemed, therefore, that the red cells circulating in the vascular system not only swelled but more cells were expelled into the system from the blood reservoirs.

Since it is known that the spleen plays a substantial part in the increment of corpuscles under many conditions (asphyxia, exercise, hemorrhage, psychological processes etc.) (2, 3), it was logical that we should remove this blood reservoir and examine a series of similar measurements on control dogs in which the spleen has been removed.

In this report, therefore, data will be presented on *a*) the water and electrolyte content in the blood and tissues of control dogs before and after splenectomy, and *b*) the changes in the equilibrium between blood and tissues in splenectomized animals following injections of large volumes of hypotonic sodium chloride solutions. These data will be compared with those obtained on control animals with spleens.

EXPERIMENTAL

Normal healthy dogs weighing 12 to 16 kg. were used for the removal of the spleen. Sodium pentobarbital anesthesia and aseptic surgical technique were used in all the operations. The dogs were maintained in metabolism cages on a diet of horse meat and Globe dog food for 3 weeks preceding the splenectomy and also following the operation until used for the injection experiments. After splenectomy, the animals were maintained and observed for different lengths of time in order that the effects of splenectomy on the concentration of the constituents of blood and tissues could be ascertained. Accordingly, the animals were divided into three groups as follows: *group 1*, 12 to 18 days after splenectomy; *group 2*, 3 to 4 weeks after splenectomy; and *group 3*, 10 to 17 weeks.

For the injection experiments all dogs were anesthetized with sodium pentobar-

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bital. The blood pressure was measured with a mercury manometer and was recorded continuously from the femoral artery throughout the injection experiment. Blood and all tissues were obtained as previously described for the control dogs with spleen (1); also the intravenous injections of 170 ml/kg. body weight of 77 mM NaCl were given in the same manner, using the same technique as for the control dogs. Since all experiments were carried out in this identical manner, comparison of blood and tissue changes in splenectomized animals could be made with those in animals with spleens.

The study included 12 splenectomized dogs, each of which was observed for the effects of splenectomy on the constituents of blood and tissues. Each dog then received an intravenous injection of a 170 ml/kg. body weight of a hypotonic saline solution consisting of 77 mM (0.45%) of NaCl.

Full details of the analytical methods and the calculations used to obtain the derived data have been given in the preceding report (1).

RESULTS AND DISCUSSION

The data are presented in two sections. The first section contains the mean values with standard deviations for serum, erythrocytes and tissues before and after splenectomy. These values show the effect of splenectomy on dogs. The second section is in turn divided into two parts. The first contains the original and derived data on control splenectomized dogs before and after subjecting them to large increases in total body water produced by the intravenous injection of hypotonic sodium chloride solutions. The second part contains a comparative histochemical interpretation of the results obtained on dogs after splenectomy before and after injections of hypotonic saline solutions, along with the parallel values on control dogs before and after the same kind of injections.

Table 1 presents the mean values for the serum and blood cell constituents of the 12 dogs. In each group the mean values of serum and cells of the dogs, after spleen removal, are given with the values of the same dogs before removal of the spleen. In *group I*, 12 to 18 days following splenectomy, it is likely that the functions of the spleen have not been entirely taken over by the other organs of the reticulo-endothelial system (liver, medullary tissue of bone, lymph nodes etc.). In this group the serum concentrations of all constituents determined did not change significantly. The hematocrit values in the 4 dogs fell from an average of 53 per cent \pm 1.6 per cent to 46 per cent \pm 3.0. The only significant difference in the concentration of the determined constituents of the red cells was the increased potassium concentration. Whether the increase in reticulocyte cells accompanying the anemia following the removal of a normal spleen as shown by Krumbhaar (4) accounts for this increase is speculative.

In *groups II* and *III*, 25 to 30 days and 75 to 120 days respectively following splenectomy, there was no change in the serum values before and after splenectomy. All animals had a lower hematocrit than their normal values throughout the 4 months observed. Pfeiffer and Smyth (5) followed patients splenectomized for traumatic rupture for 3 years and found that all patients had a distinct anemia. Krumbhaar (4) showed a post-splenectomy anemia which varied in time of appearance, degree and duration.

Although there was a decrease in the individual hematocrit values of all dogs from 12 days to 4 months post-splenectomy, the dogs still had a red cell volume within the lower limits of control values for dogs; otherwise, during these periods of observations on splenectomized dogs the water, protein and electrolyte concentrations of serum and erythrocytes did not change materially.

In table 2 are given the mean values for skeletal muscle and skin for the 12 dogs, after splenectomy, along with control data on muscle (6) and skin (1) of control dogs with spleen, which have been reported in previous papers. When the findings from the splenectomized dogs are compared with those from control dogs with

TABLE 1. MEAN VALUES FOR SERUM AND ERYTHROCYTES OF DOGS BEFORE AND AFTER SPLENECTOMY

DAYS AFTER SPLENECTOMY	NO. OF DOGS	SERUM							CELLS				
		H ₂ O	Cl	Na	K	Ca	Mg	Protein N	H ₂ O	Cl	Na	K	Cell volume
		gm.	mEq.	mEq.	mEq.	mEq.	mEq.	gm.	ml.	mEq.	mEq.	mEq.	ml. %
Group I													
0	4	913.1	108.3	140.6	4.68	5.56	1.91	10.30	701	54.0	93.0	6.41	53.4
σ±		4.7	2.2	1.5	0.32	0.15	0.04	0.07	12	3.2	2.3	0.75	1.6
12-18	4	918.9	106.2	140.1	4.72	5.50	2.20	10.31	705	59.9	94.5	9.01	45.9
σ±		3.3	2.2	1.3	0.11	0.19	0.15	1.14	9	3.9	2.8	1.1	3.0
Group II													
0	3	912.2	107.8	140.2	4.82	5.56	1.97	10.31	696	54.2	92.9	6.58	53.8
σ±		5.0	2.1	1.1	0.27	0.16	0.04	0.08	10	3.9	2.6	0.85	1.6
25-30	3	917.9	107.9	139.1	4.52	5.42	2.40	10.01	710	55.2	97.6	7.71	44.7
σ±		4.9	1.2	0.8	0.49	0.11	0.13	0.54	3	12.6	1.9	1.60	1.5
Group III													
0	5	922.2	107.2	141.4	4.75			9.64	700	62.5	94.3	9.86	51.1
σ±		2.6	2.3	0.8	1.00			0.18	12	2.2	1.7	1.34	2.0
75-120	5	924.6	108.5	142.4	4.15			9.21	716	61.7	101.8	9.47	45.3
σ±		2.4	1.5	1.6	0.72			0.50	2	2.6	5.1	1.08	2.3

The concentrations are expressed per kg. of serum and per liter of cells.

σ = Standard deviation.

spleens, the following observations are noted: *Muscle*: The total water content of the muscle was the same. There was an increased chloride of 5.57 mEq/kg. of muscle which suggests a greater extracellular volume (*F*). In fact, the average mean extracellular volume per kg. of muscle from splenectomized dogs averaged 198 gm. instead of the 154 ± 19 gm. as found in control dogs (6). This 198 gm. volume of extracellular space is significant since such an increase has been obtained experimentally when control dogs were subjected to large increases in total body water produced by the intravenous injections of 170 ml/kg. body weight of isotonic salt solution (7) or of hypotonic salt solution (1).

The increase in the extracellular phase was the result of a transference of water from the intracellular phase since the volume of intracellular water (H_2O)_o in the

splenectomized animals was 577 gm., the control value being 620 (6, 1). This lower intracellular water gives by calculation a value of 71.9 per cent for intracellular water (H_2O)_i per kg. of muscle cells, which is lower than the value of 73.6 per cent in normal muscle (6, 1). *Skin*: the total water content was approximately the same. The lowered sodium and chloride values suggest that following splenectomy there has been either a transference of water from the extracellular spaces to the cells, or sodium and chloride have been lost from the tissue. If the values are considered per 100 gm. of fat-free skin solids (table 6), the water, chloride, sodium and potassium were 272 gm., 30.4 mEq., 31.7 mEq. and 8.4 mEq., respectively, while in the control dog the values are water, 242 gm.; chloride, 30.1 mEq.; sodium, 32.9 mEq.; and

TABLE 2. MEAN VALUES FOR MUSCLE AND SKIN OF CONTROL AND SPLENECTOMIZED DOGS

	TOTAL COLLAGEN									
	H ₂ O	Cl.	Na	K	Ca	Mg	N	N	(F)	{H ₂ O} _i
	gm.	mEq.	mEq.	mEq.	mEq.	mEq.	gm.	gm.	gm.	gm.
<i>Skeletal muscle—concentrations per kg. of blood-free, fat-free tissue</i>										
Control (20).....	775.0	18.41	29.0	98.4	1.63	18.11			155	736
$\sigma \pm$	8.6	3.6	6.0	7.5	0.35	1.87			19	9
Splenectomized										
(12).....	774.8	23.98	31.10	90.8	1.88	19.46			198	719
$\sigma \pm$	4.3	4.12	4.1	4.5	0.18	1.4			36	10
<i>Skin—concentrations per kg. of fat-free tissue</i>										
Control (20).....	707.5	88.2	96.3	21.91	3.01	3.03	47.1	33.4		
$\sigma \pm$	19.8	4.9	5.7	3.5	0.52	0.37	3.7	3.0		
Splenectomized										
(12).....	725.3	83.4	87.8	22.74			44.54	32.71		
$\sigma \pm$	26.4	6.2	6.1	3.2			4.5	5.4		

σ = Standard deviation. (F) = Extracellular volume. {H₂O}_i = gm. water per kilo of muscle cells. (C) = Intracellular solids.

potassium, 7.5 mEq/100 gm. of fat-free solids. These findings indicate an increased cell water content.

When summarized, our results on values for blood and tissues of animals ranging from 12 to 120 days after splenectomy show that none of the values for the determined constituents of serum changed. In blood the percentage of 100 ml. of blood occupied by the erythrocytes or the cell volume of all dogs decreased an average of 14 per cent. In skeletal muscle, it is probable that the high values for the chloride concentration without change in total water concentration indicate an extracellular edema, the result of a transference of water from the muscle cells. In skin without a change in the total water concentration, accompanied by lowered sodium and chloride concentration, it is probable that there has been a transference of water from the extracellular spaces to the tissue cells.

The analytical results of one experiment (table 3) are shown, illustrating the

character of the experiments, the time intervals allowed, and the values both preceding (initial) and following (final) the intravenous injection of 170 ml/kg. body

TABLE 3. CHANGES IN BLOOD AND TISSUES IN SPLENECTOMIZED DOG BEFORE AND AFTER INJECTION OF HYPOTONIC NaCl SOLUTION

	HEMAT- OCRIT	RED BLOOD CELLS	pH	CO ₂	H ₂ O	Cl	Na	K	TOTAL N	COL- LAGEN N
	ml. %	millions cu. mm.		mM	gm.	mEq.	mEq.	mEq.	gm.	gm.
<i>Serum—values per kg.</i>										
Control.....					917.2	109.0	139.8	4.59	10.22	
14 days after splenec- tomy.....					918.1	108.2	138.4	4.57	10.53	
22 days after splenec- tomy.....										
Initial.....			7.37	25.82	924.9	106.7	139.5	5.21	10.01	
Final.....			4.42	17.25	949.8	104.0	129.4	4.05	6.39	
<i>Blood—values per liter</i>										
Control.....	55.3	8.10			810.7	81.8	116.6	5.30		
14 days after splenec- tomy.....	44.5	6.72			833.9	87.9	119.8	5.88		
22 days after splenec- tomy.....										
Initial.....	42.8	6.23			845.4	87.1	123.2	6.14		
Final.....	33.0	5.00			883.7	87.8	115.2	5.41		
<i>Urine per liter</i>										
						50.8	49.4			
<i>Muscle—values per kg. of blood-free, fat-free tissue</i>										
Initial.....					770.0	25.60	28.48	97.0		
Final.....					815.0	29.30	37.00	70.6		
<i>Liver—values per kg. of blood-free, fat-free tissue</i>										
Final.....					778.0	52.28	58.90	89.5		
<i>Skin—values per kg. of fat-free tissue</i>										
Initial.....					740.0	84.1	87.8	28.5	41.30	29.12
Final.....					786.0	91.0	97.1	21.5	33.00	23.54
<i>Brain—values per kg. of whole tissue</i>										
Hemisphere—final....					779.0	31.10	47.1	94.8	17.55	
Cerebellum—final....					765.0	30.27	48.5	88.2	18.10	

Solution, 77 mM NaCl.

Dog 318, control, 4/12/48, weight 16.0 kg., spleen removed; 4/26/48, blood taken; 5/3/48, weight 14.2 kg., 2400 ml. injected; urine 190 ml.

weight of a 77 mM NaCl solution to the splenectomized animal. A summary of the averages obtained from the data of all injection experiments together with the standard deviations are given in table 4. These values were used to calculate the derived

TABLE 4. AVERAGE ANALYSES OF SERUM, BLOOD CELLS AND TISSUES OF SPLENECTOMIZED DOGS BEFORE AND AFTER INJECTION

	pH	CO ₂	H ₂ O	Cl	Na	K	TOTAL N	COLLAGEN N
		mM	gm.	mEq.	mEq.	mEq.	gm.	gm.
<i>Serum—values per kg.</i>								
Initial.....	7.39	23.81	923.0	108.0	141.2	4.33	9.43	
$\sigma \pm$	0.02	1.59	3.8	1.6	1.8	0.48	0.17	
Final.....	7.40	18.15	945.0	105.9	131.3	4.13	6.17	
$\sigma \pm$	0.03	2.64	5.3	5.0	3.6	1.10	1.41	
<i>Blood cells—values per liter</i>								
Initial.....			714	60.1	99.5	8.69		
$\sigma \pm$			4	8.5	4.8	1.68		
Final.....			722	53.0	91.5	7.55		
$\sigma \pm$			12	9.0	6.3	1.73		
<i>Muscle—values per kg. of fat-free, blood-free tissue</i>								
Initial.....			774.1	23.98	31.09	90.8		
$\sigma \pm$			4.4	4.15	5.95	4.3		
Final.....			800.6	25.12	33.02	78.6		
$\sigma \pm$			8.3	4.18	4.63	9.1		
<i>Liver—values per kg. of fat-free, blood-free tissue</i>								
Initial ¹			737.3	35.91	39.5	73.2		
$\sigma \pm$			13.0	3.76	4.9	6.3		
Final.....			756.2	38.24	40.18	80.4		
$\sigma \pm$			15.8	7.65	11.2	11.9		
<i>Skin—values per kg. of fat-free tissue</i>								
Initial.....			725.0	83.4	87.6	23.00	44.55	32.26
$\sigma \pm$			26.2	8.2	6.3	3.23	4.52	5.20
Final.....			765.8	86.4	89.0	19.10	38.00	27.10
$\sigma \pm$			28.4	4.7	5.3	2.35	5.00	4.90
<i>Brain—values per kg. of whole tissue</i>								
Hemisphere								
Initial ²			761.3	36.71	51.0	95.6	18.9	
$\sigma \pm$			8.3	1.05	2.4	4.7	0.3	
Final.....			773.2	33.10	45.2	95.5	18.0	
$\sigma \pm$			7.8	1.50	4.26	6.7	0.3	
Cerebellum								
Initial ²			745.0	35.19	50.8	92.7	19.1	
$\sigma \pm$			7.0	0.89	1.7	4.0	0.5	
Final.....			758.2	31.52	48.53	90.8	17.22	
$\sigma \pm$			7.7	2.26	5.24	4.4	1.16	

 σ = Standard deviation.

Solution, 77 mM NaCl.

¹ = (8).² = (9).

data for the phase volumes of skeletal muscle shown in table 7. The normal values for liver and brain were obtained from previous studies (8, 9).

Changes in Serum and Red Cells Following Hypotonic Saline Injections. The

changes in the concentration of the constituents of the sera and red cells of the blood in the splenectomized animals following the injections were much like those found in control animals with spleens with the exception of hematocrit values and red cell counts (table 5). Contrariwise, in all the splenectomized dogs following the hypotonic saline injection, the red blood count and hematocrit values decreased. The initial values represent the ones taken on the splenectomized dog just before injection and the final values, following injection. The control values were the ones taken before splenectomy. The spleen, therefore, played a major role in the increment of corpuscles during the injection of hypotonic saline injections even though it may not be responsible for the whole phenomenon.

Changes in Tissues Following Injections. The amount of change in the concentration of constituents of all the tissues following the injection was virtually the same

TABLE 5. RED CELL AND HEMATOCRIT VALUES

DOG		CONTROL	INITIAL	FINAL
304	Red blood cells, millions/cu mm.....		7.15	5.09
	Hematocrit, %.....	52.0	47.3	35.2
305	Red blood cells, millions.....		6.13	4.86
	Hematocrit, %.....	50.0	43.5	31.7
306	Red blood cells, millions.....		5.60	5.09
	Hematocrit, %.....	53.5	41.7	35.2
314	Red blood cells, millions.....		6.81	6.31
	Hematocrit, %.....	50.9	47.6	37.3
318	Red blood cells, millions.....	8.1	6.23	5.0
	Hematocrit, %.....	55.3	42.8	33.0
320	Red blood cells, millions.....		6.90	6.45
	Hematocrit, %.....	52.0	46.6	40.8

as found in control dogs. Therefore, in splenectomized dogs the organism handled intravenous infusion of hypotonic saline in the same manner as did the control dogs with spleens. Table 6 shows comparative values of control and splenectomized dogs expressed on a dry weight basis. Although the values per 100 gm. of solids were different in the control and splenectomized dog at the start, the amount of change following the hypotonic saline injections was approximately the same. For example, in muscle the significant changes in the control animals following injections were: water, +48 gm.; chloride, +1.94 mEq.; sodium, no change; in the splenectomized animals the changes were: water, +57 gm.; chloride, +1.94 mEq.; sodium, +2.68 mEq. In skin, the significant changes in the control dog were: water, +69 gm.; chloride, +5.0 mEq.; sodium, +5.0 mEq.; in the splenectomized animals the changes were: water, +68 gm.; chloride, +6.8 mEq.; and sodium, +7.2 mEq.

The values obtained on brain (table 6) show that there has been an increase in

water per 100 gm. of solids without accompanying changes in chloride or sodium concentration. This suggests that the extra water of the brain was intracellular.

TABLE 6. AVERAGE ANALYSES EXPRESSED ON DRY WEIGHT BASIS OF TISSUES OF CONTROL AND SPLENECTOMIZED DOGS BEFORE AND AFTER INJECTION

	H ₂ O	Cl	Na	K	TOTAL N	COLLAGEN N
	gm.	mEq.	mEq.	mEq.	gm.	gm.
<i>Muscle—values per 100 gm. fat-free, blood-free solid</i>						
Control.....	328	8.87	11.26	41.3		
$\sigma \pm$	6	0.95	2.02	3.2		
Hypotonic.....	376	10.56	11.12	39.6		
$\sigma \pm$	12	1.76	2.91	3.3		
Splenectomized.....	346	10.73	14.00	40.4		
$\sigma \pm$	8	1.97	2.88	1.3		
Hypotonic.....	403	12.67	16.68	39.4		
$\sigma \pm$	23	2.13	2.86	3.4		
<i>Skin—values per 100 gm. fat-free solid</i>						
Control.....	243	30.7	33.0	7.37	16.2	11.5
$\sigma \pm$	28	2.0	2.3	2.09	0.3	0.8
Hypotonic.....	312	35.7	38.0	7.50	16.2	11.5
$\sigma \pm$	37	2.9	4.3	2.22	0.3	0.6
Splenectomized.....	272	30.4	31.7	8.43	16.2	11.7
$\sigma \pm$	40	1.8	2.7	1.17	0.3	1.1
Hypotonic.....	334	37.2	38.9	8.30	16.2	11.5
$\sigma \pm$	45	2.7	4.1	1.58	0.3	0.9
<i>Brain—values per 100 gm. whole tissue solid</i>						
Hemisphere						
Control.....	319	15.34	21.38	39.9	7.92	
$\sigma \pm$	14	0.79	1.51	3.0	0.18	
Hypotonic.....	351	14.91	21.26	41.5	7.97	
$\sigma \pm$	22	0.85	1.11	1.7	0.38	
Splenectomized						
Hypotonic.....	342	14.66	21.25	42.3	7.94	
$\sigma \pm$	14	0.82	1.37	3.9	0.13	
Cerebellum						
Control.....	292	13.82	19.90	36.4	7.45	
$\sigma \pm$	14	0.33	0.90	0.8	0.20	
Hypotonic.....	321	12.98	20.11	38.1	7.53	
$\sigma \pm$	10	0.62	1.00	1.6	0.25	
Splenectomized						
Hypotonic.....	314	13.02	20.07	37.7	7.11	
$\sigma \pm$	14	0.87	1.95	2.4	0.20	

σ = Standard deviation. Solution, 77 mM NaCl.

It will be noted that the hemisphere and cerebellum values for the constituents determined following the hypotonic saline injections are approximately the same in both the control and splenectomized animals.

For purposes of comparison, the data for phase volumes of muscle and liver from all animals have been grouped in table 7. Description of the table is given in the legend appended. The derived data were calculated from the experimental results given in table 4. It will be noted that following large injections of hypotonic sodium chloride in the splenectomized animal the original kilogram of skeletal

TABLE 7. PHASE VOLUME DATA FROM ALL DOGS, GROUPED FOR PURPOSES OF COMPARISON

	NO. OF ANI-MALS	T		(F)		(H ₂ O) _e		(S)		{H ₂ O} _e	
		Mean	σ±	Mean	σ±	Mean	σ±	Mean	σ±	Mean	σ±
<i>Skeletal Muscle</i>											
Normal Animals											
Before injection.....	10 ¹	1000		169	18	596	18	235	3	718	8
After injection.....	8 ²	1110	32	213	34	663	28	235	3	738	7
Δ.....		+111		+44		+67				+20	
Splenectomized Animals											
Before injection.....	10	1000		198	36	577	34	225	4	719	11
Δ N.....				+29		-19		-10		+1	
After injection.....		1133	52	250	46	659	31	225	4	744	9
Δ.....		+133		+52		+82				+25	
Δ N ₁		+23		+37		-4		-10			
<i>Liver</i>											
Normal Animals											
Before injection.....	10 ¹	1000		294	65	443	67	263	13	628	22
After injection.....	8 ²	1152	65	311	54	578	65	263	13	687	47
Δ.....		+152		+17		+135				+59	
Splenectomized Animals											
After injection.....		1109	45	366	53	480	56	263	13	638	28
Δ N ₁	10	-43		+55		-98				-49	

¹ = (8). ² = (1).

(F) = gm. of extracellular phase per kg. of tissue. (H₂O)_e = gm. of intracellular water per kg. of tissue. (S) = Solids of intracellular phase. T = (F) + (H₂O)_e + (S). {H₂O}_i = gm. of water per kg. of tissue cells. Δ = Differences between means before and after injection. Δ N = Differences between means of control animals and of splenectomized animals before injection. Δ N₁ = Differences between means of control animals and of splenectomized animals after injection. σ = Standard deviation.

All values except {H₂O}_i are expressed per kg. of fat-free, blood-free tissue.

Solution injected, 77 mM NaCl.

muscle increased to 1133 gm. of muscle, of which 51 gm. are attributed to the extra-cellular spaces and 82 gm. to the tissue cells, which is about the same percentage of change that was obtained in control dogs with spleens; also the percentage of water per kilogram of muscle cells increased from 71.9 to 74.4 per cent in the splenectomized dogs, which is about the same as found in the control dogs. It is regrettable that values for the constituents of liver from splenectomized animals before injec-

tions were not established. When the liver values from the splenectomized animals after injection are compared with the values obtained on control dogs after injection, the liver cells did not swell as much in the animals after splenectomy.

Table 8 gives the urinary excretion of water, sodium and chloride during the experimental period. It will be noted that the dogs excreted a urine with a Na:Cl ratio of 1.0, the same ratio as found in control dogs (1). The urinary loss of water, sodium and chloride minus the water, sodium and chloride injected is given in the column under load, and the loads are expressed in terms of concentration per liter, as given in the last column. These splenectomized animals, the same as was found in the animals with spleens, retained a hypotonic salt solution ranging from 74 to 82 mEq. per liter. Thus, the removal of the spleen from the dog had no effect upon the excretion of water, sodium and chloride following the hypotonic saline injections.

It may be concluded, therefore, that animals without spleens distribute the increases in total body water in the tissues following hypotonic saline injections the same as control dogs with spleens. The decrease in the red cell volume and

TABLE 8

DOG	SALINE INJECTION	URINE	WATER LOAD	Na AND Cl INJECTED	Cl EXCRETED	Cl LOAD	Na EXCRETED	Na LOAD	RETENTION CONCENTRATION
	ml.	ml.	ml.	mEq.	mEq.	mEq.	mEq.	mEq.	mEq/l.
303	2400	370	2030	185	35	150	34	151	74-75
304	1880	210	1670	145	18	127	19	126	76-75
305	2300	350	1950	177	27	150	28	149	77-77
306	2620	415	2205	201	20	182	19	182	82-82
313	2148	385	1763	165	30	135	27	138	77-78
314	2300	460	1840	177	38	138	38	139	75-75

counts following the injections and producing an anoxemia may have other physiological effects not considered here. The fact that control dogs with spleens expelled extra red blood cells into the vascular system while receiving hypotonic saline injections under these experimental conditions whereas the splenectomized animals did not, substantiates further the fact that the spleen is an important blood reservoir.

SUMMARY

The effects of splenectomy on the concentration of water and electrolytes of the blood and tissues were determined over a period of from 12 to 120 days following splenectomy. After splenectomy, no observed changes were found in the concentration of constituents in the serum and red blood cells. There was a decrease in the red cell volume in all dogs. In skeletal muscle, moderate changes were found in the distribution of water in the tissue: an increase in the extracellular volume at the expense of the cellular mass. In the skin, changes in gross composition involved a lowered sodium and chloride concentration, which probably indicates a transference of water from the extracellular spaces to the cells.

After subjecting the splenectomized dogs to large increases in total body water

produced by the intravenous injection of hypotonic sodium chloride solution, it was noted that 1) the red cell volume and red cell count fell markedly, but 2) the distribution of the extra water and electrolytes in blood and tissues was the same as found in the control dogs with spleens. Thus, in the splenectomized as well as in control animals the decrease in sodium concentration in the body fluids caused a shift of the extra water into cells of the body.

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RELATION OF GLOMERULAR FILTRATION RATE AND SODIUM TUBULAR REJECTION FRACTION TO RENAL SODIUM EXCRETION¹

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RENAL regulation of sodium output is presumably mediated through the rate of sodium filtration and the fraction of filtered sodium which escapes tubular reabsorption.

One can conceive of two extreme situations in such regulation: if the fraction rejected by the tubule were constant, sodium output would be fixed by filtration rate, and an exact linear relation would exist between the two parameters. Conversely, if the filtration rate were constant, sodium excretion would be perfectly correlated with the fraction rejected. Variability in both filtration rate and rejection fraction would provide an intermediate situation in which one or other of these functions would be the major, but not sole renal agent for sodium output regulation.

The object of the present study was to determine which of these situations best accorded with observations of human sodium output relationships. The question of particular interest was not why one subject excreted more sodium than another but how it was accomplished, and whether or not the observed differences in output could be primarily explained by variations in filtration rate, or by differences in tubular reabsorption.

In normal dogs, the sodium output has been increased as much as several hundred-fold by imposition of varying sodium loads (1). In this species the rate of glomerular filtration is relatively flexible. It can be altered by extremes of hydration, as well as by the quantity of dietary protein (2). Nevertheless, these changes in sodium output were not associated with consistent alterations in glomerular filtration rate. On the contrary, they were effected almost entirely by variations in tubular reabsorption.

In man, the filtration rate is more stable. It fluctuates little in any one individual in response to diet (3), hydration (4), the pyrogenic reaction or adrenaline (5). Between individuals, great differences may exist as a consequence of renal and cardiovascular disease. The subject group for the present study was selected so as to provide a cross-section of glomerular filtration rates. The dietary sodium intakes were sufficiently varied so that wide differences in sodium output could be anticipated within the group.

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MATERIAL AND METHODS

The subjects consisted of 60 hospital patients, the majority of whom were suffering from essential hypertension, glomerulonephritis, arteriosclerotic heart disease or toxemia of pregnancy. None of the patients was edematous. Ten were maintained on diets which contained approximately 2 gm. of sodium chloride per day. The remainder used salt *ad libitum*. No foods or liquids were allowed during the 12 hours preceding study.

The magnitude of sodium filtration was estimated in all subjects by the expression:

$$GFR_{Na} = GFR \times P_{Na}$$

where: GFR_{Na} = Sodium filtration rate (mEq/min.)

GFR = Glomerular filtration rate (cc/min.) as measured by the mannitol technique (6)

P_{Na} = Plasma sodium concentration (mEq/cc.) as measured by the uranium acetate method (7)

The priming dose of mannitol, 8 gm/m², was administered as a 25 per cent solution in water. The sustaining dose, 75 mg/minute/m², was made up with sodium chloride and water to yield an approximately isosmotic solution.

The influence of tubular activity on sodium excretion was estimated in all subjects by the expression:

$$TRF_{Na} = \frac{U_{Na} \cdot V}{GFR_{Na}} \times 100,$$

where: TRF_{Na} = Sodium tubular rejection fraction (mEq. of sodium excreted per 100 mEq. filtered)

U_{Na} = Urinary sodium concentration (mEq/cc.)

V = Urine flow (cc/min.)

GFR_{Na} = Sodium filtration rate (mEq/min.)

The variation of any measured factor was estimated by the standard deviation and coefficient of variation. The degree of linear correlation between two factors was established by use of the correlation coefficient (r_{xy}). The net relationship between two factors when a third interdependent factor was held constant was estimated by calculation of the coefficient of partial correlation ($r_{xy,z}$).

RESULTS

The sodium output during study averaged 0.229 mEq/minute/m².² The water output was 2.67 cc/minute/m². These relatively high values appeared due in part to the daytime tide of urine flow (8), as well as to the constant load imposed by the test solutions, which amounted to 0.100 mEq. of sodium and 1.07 cc. of water/minute/m². A less constant influence was presented by the osmotic activity of mannitol in the tubular urine (9), since the rate of its excretion was necessarily related to the glomerular filtration rate ($r_{xy} = .847$).

² For detailed table, order Document No. 2712 from American Documentation Institute, 1719 N Street, N.W., Washington 6, D. C., remitting 50 cents for microfilm (images one inch high on standard 35-mm. motion picture film) or 70 cents for photocopies (6 × 8 inches) readable without optical aid.

In view of this variation, the question arose as to the applicability of sodium output relationships established during clearance determinations involving the use of mannitol. The output of mannitol during these studies averaged 87.2 mEq/l. Since the osmolarity of normal urine is a variable, which averages perhaps 760 mEq/l. (10), about seven eighths of which is due to constituents other than sodium, it seemed likely that the sodium excretion rates measured in this study were influenced by normal osmotic factors several times as great as the mannitol factor. The pertinent question appeared to be whether or not the differences in sodium output observed among the subjects of this study had been influenced significantly by differences in mannitol output. If such were the case one might anticipate a comparable range of

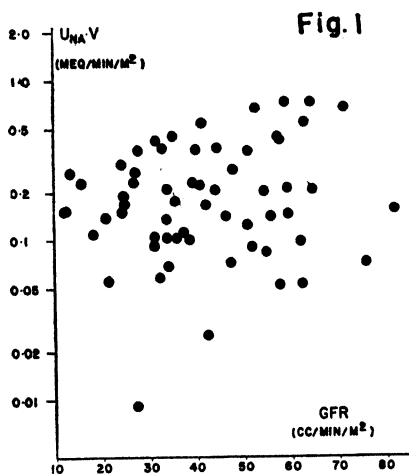


Fig. 1. RELATION of sodium excretion to glomerular filtration rate.

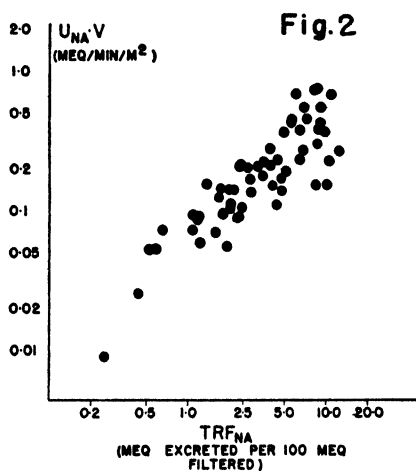


Fig. 2. RELATION of sodium excretion to sodium tubular rejection fraction.

variation for the two factors and significant correlation between the excretion of mannitol and that of sodium.

Examination of the data indicated that the highest sodium excretory rate was more than eighty times the lowest, the coefficient of variation being .782. The highest rate of mannitol excretion was less than eight times the lowest, the coefficient of variation being .376. Calculation of the coefficient of correlation failed to demonstrate the existence of a significant association between mannitol and sodium outputs ($r_{xy} = .168$; $t = 1.30$).

On the basis of these findings it did not appear that the quantities of mannitol used had been sufficiently large and variable to have accounted for the differences in sodium excretion observed within the subject group.

In order to determine whether or not these differences were referable to variations in filtration rate, a comparison of the two parameters was made for the entire group (fig. 1). No significant correlation was demonstrated ($r_{xy} = .243$; $t = 1.91$). Correlation between filtration rate and sodium output, holding the mannitol output constant, was even lower ($r_{xy.s} = .208$; $t = 1.62$). Sodium outputs varied from 0.009

to $0.735 \text{ mEq/minute/m}^2$, a range nearly twelve times that of the filtration rate, which varied from 12.1 to $82.0 \text{ cc/minute/m}^2$. Ten- to 50-fold differences in sodium excretion were manifested by different subjects at nearly identical rates of filtration (and, as a corollary, of mannitol output).

In contrast, a high degree of association was found in a comparison of sodium excretion with sodium tubular rejection fraction (fig. 2). The range of the latter parameter extended from 0.25 to 12.50 per cent of the amount of sodium filtered. The coefficient of variation ($V = .723$) was comparable with that for sodium output ($V = .782$). The coefficient of correlation between the two factors ($r_{xy} = .708$) proved to be highly significant ($t = 7.64$). When the mannitol output was held constant, the correlation was still greater ($r_{xy, z} = .829$; $t = 11.30$).

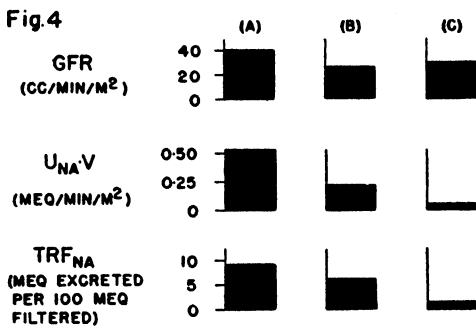
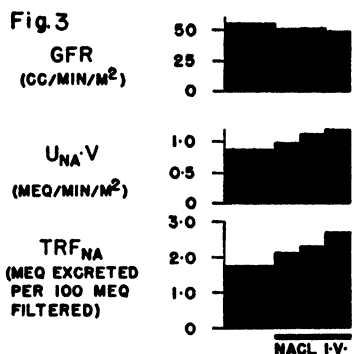


Fig. 3. EFFECTS OF SODIUM LOAD ON SODIUM EXCRETION of a human subject during 3 successive clearance periods. Rate of administration, $0.80 \text{ mEq/minute/m}^2$.

Fig. 4. FILTRATION RATE, TUBULAR REJECTION FRACTION AND SODIUM OUTPUT of one subject at three different levels of sodium exchange (see text).

The relation of the tubular rejection fraction to sodium excretion may be further illustrated by the results of two additional experiments. In the first (fig. 3) the subject was infused with isosmotic sodium chloride solution for one hour at a rate of $0.80 \text{ mEq/minute/m}^2$. By the end of the period, the output of sodium had increased from 0.088 to $0.119 \text{ mEq/minute/m}^2$. Since the filtration rate had decreased slightly from the control value, the rise in sodium excretion was accomplished by an increase of over 50 per cent in the sodium tubular rejection fraction.

In the second experiment, the filtration rate and sodium output of the same subject were measured at 3 different levels of sodium exchange (fig. 4). The filtration rate during the 2 latter determinations was approximately two-thirds of the value found during the initial measurement. This difference appeared consistent with the subject's changing cardiovascular status. The sodium output during the period in which the subject was allowed salt *ad libitum* was $0.539 \text{ mEq/minute/m}^2$. When all free salt was eliminated from the diet, the output dropped to $0.230 \text{ mEq/minute/m}^2$. Three days after a mercurial diuretic had been given to facilitate sodium depletion, the rate of excretion had fallen to $0.063 \text{ mEq/minute/m}^2$. The highest excretory rate was thus more than eight and one-half times greater than the lowest. The differences in sodium

output were too great to be explained by the relatively small alterations in filtration rate. They appeared to have been accomplished primarily through the agency of comparable variations in tubular rejection fraction.

SUMMARY AND CONCLUSIONS

The mechanism of sodium excretion was studied in 60 subjects. The group was selected to provide a representative cross-section of filtration rates. Dietary sodium intake was sufficiently varied to insure a wide range of sodium excretion rates. The objective of the study was not to determine why one subject excreted more sodium than another, but to ascertain whether or not the observed differences in output could be primarily explained by differences in glomerular filtration rate or by variations in tubular reabsorption. Under the conditions of the study, a high correlation was found between the rate of sodium excretion and the magnitude of the tubular rejection fraction. No significant relation to filtration rate was established. Ten- to 50-fold variations in sodium output were observed at nearly identical rates of filtration. No evidence was found that the relationships observed had been significantly influenced by the use of mannitol in the clearance measurements.

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RENAL TUBULAR ELIMINATION OF N¹-METHYLNICOTINAMIDE¹

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IT IS well established that the cells lining the renal tubules are capable of both reabsorptive and secretory functions, which together with glomerular filtration are responsible for the elaboration of urine. It is equally well known that several functionally discrete reabsorptive mechanisms that are non-competitive, i.e. for the reabsorption of glucose, certain amino acids etc., exist. Moreover, these mechanisms can be shown to be non-competitive as functional entities but are subject to the same fundamental principles that govern the affinities of substrates for certain enzyme systems; at least this is true insofar as they have been studied carefully.

On the other hand, only one functionally discrete renal tubular secretory mechanism has been demonstrated unequivocally since the classic studies by Marshall and his associates on the excretion of phenol red (1-3), unless one considers the excretion of exogenous creatinine by man (4-6) and the recent interesting studies on potassium excretion (7, 8) to belong in this general category. Except for this latter reservation, all organic compounds that have been found to be secreted by the renal tubules have been shown to compete on a mass action basis with each other and with phenol red for elimination by this mechanism. These have included such diverse compounds as certain pyridones, hippurates (9, 10), and the penicillins (11). In general these compounds have been acids.

Sperber first called to our attention his observation that N¹-methylnicotinamide (NMN) appeared to be secreted by the mesonephric kidney of the fowl (12). It was this observation that stimulated our evaluation of the excretory components involved in the elimination of this compound by the metanephric kidney of mammals.

N¹-methylnicotinamide is a principal metabolic product of nicotinic acid (13). Since it is excreted following the administration of either the acid or nicotinamide (14) and since nicotinamide, not nicotinic acid, has been shown to be transmethylated in mammals (15), this pathway, rather than, as assumed by some authors, nicotinuric acid (16), seems to be the principal one for the metabolism of nicotinic acid.

The purpose of this communication is to describe experiments the results of which may be interpreted to indicate that NMN is secreted by a second tubular transport mechanism that is distinct functionally from that presently recognized. The identity of this second secretory mechanism has been supported by 1) the behavior of the excretory pattern for this basic compound, which is characteristic of agents secreted by the tubules, 2) by the observation that it does not suppress the tubular secretion of p-aminohippurate and, conversely, 3) by the finding that p-aminohippurate does not block the tubular elimination of N-methylnicotinamide.

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METHODS

Seven well trained female dogs were used in the course of these clearance experiments. Two of these animals were prepared for the determination of simultaneous renal clearances and extraction ratios by the removal of one kidney and the explanation of the opposite organ under the skin of the flank, according to the procedure of Page and Corcoran (17). Femoral arterial and renal vein blood samples were obtained for the renal extraction studies. In several experiments sufficient plasma was obtained to permit the determination of the ultrafilterable concentration of NMN in plasma, using the method of Lavietes for obtaining plasma ultrafiltrates (18).

The NMN contents of plasma and urine were determined by the method of Huff and Perlzweig (19). An aliquot of the filtrate from a 1:5 dilution-trichloroacetic acid-precipitation of plasma was portioned into three 1.0 cc. aliquots set up in fluorimeter tubes. To the first was added acetone (0.5 cc.) and water (0.5 cc.); to the second was added 1.0 cc. of water; and to the third was added acetone (0.5 cc.) and 5 micrograms of NMN (0.5 cc.) as an internal standard. Thereafter the procedure was the same in all three tubes. The urine samples were diluted 1:10 beyond the dilution introduced for creatinine determinations. All final dilutions contained 0.4 cc. of glacial acetic acid per 20 cc. The urine samples were decolorized with charcoal, filtered, and analyzed immediately. These filtrates were set up as described for plasma. Appropriate plasma and urine control blank determinations were performed. The instrument used was a Klett Fluorimeter, Model 2070. Analytical methods for creatinine, p-aminohippurate (PAH), and carinamide² were standard ones and have been referred to previously in publications from this laboratory. Nicotinamide was determined by the method of Snell and Wright (20). Preliminary determinations were made in each instance to establish the absence of interference of one compound with the determination of another in plasma and urine at the approximate concentrations used in the clearance experiments.

RESULTS

As the plasma concentration of NMN was elevated, its renal clearance decreased to approach that of glomerular filtration rate (creatinine clearance) as a limit. Under no circumstance have we found the clearance of NMN to equal or be less than glomerular filtration rate. Table 1 illustrates this generalization and serves to present the basic design of the experiments, other than those wherein simultaneous renal extractions and clearances were performed. Also, it illustrates certain other points pertaining to the properties of NMN.

The depression of renal clearance and the clearance ratio of NMN/creatinine are similar to those observed for other compounds secreted by the tubules. This is due to the fact that, whereas the amount filtered per unit time is determined by the load or ultrafilterable plasma concentration at any given glomerular filtration rate, the amount secreted by the tubules per unit time is determined by the overall rate at which the cells can extract or secrete the compound, and is independent of load

² Carinamide is the non-proprietary name for 4'-carboxyphenylmethanesulfonanilide. Formerly the spelling of this name was caronamide. 'Statin' is the trademark applied to this compound by Sharp and Dohme, Inc.

when the functional capacity (T_m) of the transport system has been exceeded. Smith, Goldring, and Chasis have demonstrated this effect for phenol red, 'Diodrast,' and 'Hippuran,' and have discussed the phenomenon at some length (9). This self-depression of clearance is common as well to PAH, as will be illustrated in the following data (tables 3 and 4), and to the penicillins (21).

We have not attempted to determine carefully the secretory capacity (T_m) for NMN since at high dosages it is toxic, producing in some instances nausea and vomiting, frequently a reduction in glomerular filtration rate and/or urine flow, and invariably hemolysis and hematuria. The occurrence of these toxic effects of cre-

TABLE 1. RELATIONSHIP OF NMN PLASMA CONCENTRATION TO ITS RENAL CLEARANCE
Dog 365, wt. = 14.4 kg.

TIME	URINE FLOW	CREATININE CLEARANCE	N ¹ -METHYLNICOTINAMIDE (NMN)		
			Plasma Conc.	Clearance (UV/P)	Clearance ratio
hr.:min.	cc/min.	cc/min.	γ /cc.	cc/min.	
0:00	500 cc. water, p.o.				
1:00	300 cc. water, p.o.				
1:15	Bladder catheterized, urine discarded				
1:20	Control blood sample				
1:25	Control 10-minute urine sample				
1:28	3.0 gm. creatinine, s.c.				
1:30	NMN Priming dose 0.25 mg/kg. (3.6 mg. total), i.v.				
	NMN Venoclysis 0.25 mg/kg/min. at 3 cc/min.				
1:45-55	4.7	63.94	5.9	156.22	2.44
1:55-65	4.2	58.23	6.9	134.29	2.31
2:10	NMN Priming dose 1.0 mg/kg. (14.4 mg. total), i.v.				
	NMN Venoclysis 1.0 mg/kg/min. at 3 cc/min.				
2:30-40	3.1 ¹	47.28	48.9 ¹	67.21	1.42
2:40-50	3.2 ¹	47.72	62.8 ¹	63.78	1.34

¹ Hemolysis, hematuria.

atinine clearance depression, hemolysis and hematuria, is illustrated by the data in table 1.

Table 2 summarizes the data for the simultaneous renal extraction and clearance of NMN in an experiment on a dog that had a unilateral nephrectomy and a contralateral renal explant. Here it may be seen that, compared to creatinine, the extraction ratio and clearance ratio were greater than 1.0 and were in good agreement. This would indicate that the drug was secreted by the tubules and that no determinable amount was retained progressively in the cells for synthesis or destruction.

The lack of binding of NMN on plasma protein is illustrated by the data in table 2, although perhaps not as well as is demonstrated in less complicated experiments. However, it serves to support the conclusion drawn from additional data.

The lack of effect of NMN on the renal clearance and tubular secretory capacity (T_m) for *p*-aminohippurate is illustrated in tables 3 and 4. Table 3 presents average data from an experiment wherein, following duplicate control clearances, the effect

TABLE 2. SIMULTANEOUS RENAL EXTRACTION AND CLEARANCE OF NMN BY DOG HAVING RIGHT UNILATERAL NEPHRECTOMY AND LEFT RENAL EXPLANT OF ABOUT SIX MONTHS' DURATION

URINE FLOW	CREATININE		N ¹ -METHYLNICOTINAMIDE				
	Percentage extracted	Clearance	Plasma conc.		Percentage extracted	Clearance	Extraction ratio
			Artery	Vein			
cc/min.		cc/min.	γ /cc.			cc/min.	
2.2	30.61	39.0	Dog 1005N, wt. = 18.1 kg.				
			16.3 ¹	5.0 ¹	69.3	85.0	2.26
							2.20

In this experiment the NMN was administered as a priming dose of 0.25 mg/kg. i.v. followed by a venoclysis of 0.25 mg/kg/min. at a rate of 3 cc/min.

¹ Following ultrafiltration by the method of Lavietes (*J. Biol. Chem.* 120: 267, 1937), the arterial and venous plasma concentrations were 14.6 and 5.2 mg/100 cc. of ultrafiltrate.

TABLE 3. DEMONSTRATING LACK OF COMPETITIVE INHIBITION OF PAH CLEARANCE BY NMN

TIME	URINE FLOW	CREATININE CLEARANCE	P-AMINOHIPPURATE		
			Plasma Conc.	Clearance	Clearance ratio
hr.:min.	cc/min.	cc/min.	γ /cc.	cc/min.	
0:00	500 cc. water, p.o.				
1:00	PAH 150 mg/kg. in 200 cc. water, p.o.				
1:50	3.0 gm. creatinine, s.c.				
2:20-30	3.2	50.60	8.1	158.54	3.13
2:30-40	0.5	43.00	8.9	125.39	2.92
2:45	NMN Priming dose 1.0 mg/kg., i.v. Venoclysis 1.0 mg/kg/min. at 3 cc/min.				
3:00-10	2.3	46.68	8.3	136.06	2.91
3:10-20	2.2	48.83	7.7	140.55	2.88
			NMN		
3:00-10			60.2	56.03	1.20
3:10-20			57.3	56.58	1.16

of NMN at high plasma concentration on the clearance of PAH at very low plasma concentrations (0.81 mg/100 cc.) was determined. There was no decrease in PAH clearance or in PAH/creatinine clearance ratio.

In the experiment illustrated in table 4, the plasma concentration of PAH was elevated to the point that its tubular secretory capacity (T_m) was determined before and following the coadministration of NMN. In this and other experiments,

wherein the concentration of NMN was higher, there was no suppression of the T_m for PAH. Thus NMN does not compete with PAH for elimination by its transport mechanism nor does NMN appear to decrease the functional capacity of that particular secretory process.

PAH did not inhibit the clearance or the clearance ratio of NMN/creatinine even when the plasma concentration of PAH was sufficient to overload the tubular secretory capacity for that compound. This is illustrated by an experiment summarized in table 5 wherein duplicate NMN clearances were performed with and with-

TABLE 4. DEMONSTRATING LACK OF EFFECT OF NMN ON TUBULAR FUNCTIONAL CAPACITY (T_m) FOR SECRETION OF PAH

Dog 58, wt. = 15.6 kg.

TIME	URINE FLOW	CREATININE CLEARANCE	P-AMINOHIPPURATE			
			Plasma Conc.	Clearance	Clearance ratio	T _m
<i>hr.:min.</i>	<i>cc/min.</i>	<i>cc/min.</i>	<i>mg/100 cc.</i>	<i>cc/min.</i>		<i>mg/min.</i>
1:45	PAH Priming dose 400 mg/kg. i.v.					
1:47	Venoclysis 125 mg/kg/hr. in 5% glucose at 3 cc/min.					
	Creatinine 3.0 gm. s.c.					
2:15-2:25	2.9	48.14	28.38	86.5	1.8	13.20
2:25-2:35	2.4	52.24	26.16	92.4	1.8	12.85
2:40	PAH Venoclysis continued					
	NMN Priming dose 0.25 mg/kg. i.v.					
	Venoclysis 0.25 mg/kg/min. (added to PAH infusion solution).					
3:10-3:20	3.5	57.85	24.42	106.7	1.8	14.34
3:20-3:30	3.2	52.16	23.98	105.4	2.0	14.81
			NMN			
3:10-3:20			9.00 ¹	104.3	1.8	
3:20-3:30			10.40 ¹	128.2	2.4	

¹ Plasma conc. NMN expressed in γ/100 cc.

out the coadministration of PAH in sufficient amounts to exceed its T_m. Although the experiments actually show a slight rise in the clearance ratio of NMN/creatinine, this has no significance and was not substantiated in other experiments. Thus, from the experiments represented in tables 3, 4 and 5, it may be concluded that NMN is secreted by a tubular transport mechanism that is distinct from that for the secretion of PAH.

Carinamide did not inhibit the clearance of NMN when administered under the same conditions in which it suppressed completely the tubular secretion of penicillin (22) and markedly depressed the clearance of PAH (23). This observation is illustrated by the data presented in table 6. The priming and infusion dosages for carinamide were the same as in the experiments referred to in the previous sentence.

TABLE 5. DEMONSTRATING LACK OF COMPETITIVE INHIBITION OF NMN CLEARANCE BY PAH

Dog 472, wt. = 11.8 kg.

TIME	URINE FLOW	CREATININE CLEARANCE	N ¹ -METHYLNICOTINAMIDE		
			Plasma Conc.	Clearance	Clearance ratio
<i>hr.:min.</i>	<i>cc/min.</i>	<i>cc/min.</i>	<i>γ/cc.</i>	<i>cc/min.</i>	
1:30	NMN Priming dose 0.25 mg/kg. Venoclysis 0.25 mg/kg/min. at 3 cc/min.				
1:45-55	4.9	48.16	8.6	84.64	1.76
1:55-65	4.6	45.67	10.2	82.77	1.81
2:10	PAH Priming dose 100 mg/kg. Venoclysis 100 mg/kg/hr. NMN Venoclysis 0.25 mg/kg/min. at 3 cc/min.				
2:35-45	5.9	44.96	14.9	99.79	2.22
2:45-55	5.1	37.16	15.9	99.80	2.69
			PAH ¹		
2:35-45			46.2	76.11	1.45
2:45-55			40.5	81.09	1.61

¹ Plasma concentration mg/100 cc.

TABLE 6. LACK OF INHIBITORY EFFECT OF CARINAMIDE ON RENAL CLEARANCE OF NMN

Dog 370, wt. = 15 kg.

TIME	URINE FLOW ¹	CREATININE CLEARANCE	N ¹ -METHYLNICOTINAMIDE		
			Plasma ¹ Conc.	Clearance	Clearance ratio
<i>hr.:min.</i>	<i>cc/min.</i>	<i>cc/min.</i>	<i>γ/cc.</i>	<i>cc/min.</i>	
1:30	NMN Priming dose 0.5 mg/kg. Venoclysis 0.5 mg/kg/min. at 3 cc/min.				
1:45-55	4.7	70.20	30.4	95.71	1.36
1:55-65	4.0	64.00	30.0	87.23	1.36
2:10	Carinamide Priming dose 25 mg/kg. Venoclysis 0.5 mg/kg/min. NMN Venoclysis 0.5 mg/kg/min.				
2:25-35	3.8	64.02	32.0	114.83	1.79
2:35-45	2.9	54.78	43.6	73.50	1.34

¹ Progressive hemolysis and hematuria.

Under these conditions the plasma concentration of carinamide was about 8 mg/100 cc.

Although carinamide is not secreted by the renal tubules (24-26), it does have

an affinity for, and can inhibit, the transport mechanism responsible for the secretion of phenol red, PAH, and penicillin (22, 23). The observation that carinamide did not inhibit the tubular secretion of NMN indicates 1) that NMN is secreted by a mechanism that is distinct from the aforementioned one for PAH elimination and 2) that the inhibitory effect of carinamide is on one, but certainly not on all, tubular secretory mechanisms.

After the lack of a cross-inhibition of the two tubular secretory mechanisms was demonstrated, related derivatives of nicotinic acid were studied for an inhibitory action on the excretion of NMN.

None of the nicotinic acid derivatives studied inhibited the tubular secretion of NMN. These compounds included nicotinamide, 1-methyl-3-carboxylamide-6-pyridone, and trigonelline. Trigonelline is N-methylnicotinic acid. Neither of the latter two compounds was determined in plasma or urine during the experiments. The data for these several experiments are included in tables 7 and 8.

It may be of interest to point out the very low clearance of nicotinamide and the fact that NMN did not alter its clearance ratio significantly (see table 7). The dosage of the three compounds was adjusted to the maximal amount we considered feasible to administer, on the basis of toxicity, availability, solubility, etc.

COMMENT

On the basis of data from which the illustrative material for this communication was drawn it is our conclusion that the renal elimination of N¹-methylnicotinamide is brought about, in addition to glomerular filtration, by a tubular secretory transport mechanism that is distinct, functionally, from that responsible for the secretion of hippurates, penicillins, and certain pyridones. It is interesting that, like PAH, NMN is not bound on plasma protein to any great extent. These two compounds represent a fairly strong organic acid and base, respectively. The fact that they differ so in this property makes it the more probable that, since both are secreted by the tubules, the transport mechanisms involved in their cellular secretion should be different, assuming that at least two distinct processes are available. In essence, NMN may be taken as an example of compounds secreted by a system limited to bases, possibly quarternary bases, just as the group of compounds secreted by the other system mentioned seems to be reserved to acids. However, this interpretation goes beyond the data and can only serve as an interesting speculation.

It is of more than academic interest that carinamide can be shown not to block all tubular secretion. Since it does not inhibit the tubular secretion of NMN this evidence serves to strengthen the position that carinamide blocks a specific tubular transport process and not tubular secretion in general.

It is not surprising that nicotinamide does not inhibit the tubular secretion of NMN, for the former is a tertiary amine and the latter is a quarternary ammonium compound. Moreover, nicotinamide is reabsorbed in large measure by the tubules whereas NMN is secreted thereby; thus, there is a qualitative difference in the manner in which the kidney handles the two compounds.

It is interesting that neither trigonelline nor 1-methyl-3-carboxylamide-6-pyridone influenced the renal elimination of NMN perceptibly, although their plasma

TABLE 7. LACK OF INHIBITOR EFFECT OF NICOTINAMIDE ON RENAL ELIMINATION OF NMN

Dog 370, wt. = 16.0 kg.

TIME	URINE FLOW	CREATININE CLEARANCE	NICOTINAMIDE			N ¹ -METHYLNICOTINAMIDE		
			Plasma Conc.	Clearance	Clearance ratio	Plasma Conc.	Clearance	Clearance ratio
	<i>cc/min.</i>	<i>cc/min.</i>	<i>γ/cc.</i>	<i>cc/min.</i>		<i>γ/cc.</i>	<i>cc/min.</i>	
	Nicotinamide Endogenous control							
1:50	Creatinine 3.0 gm. s.c.							
1:55	NMN Priming dose 0.25 mg/kg. i.v.							
	Venoclysis 0.25 mg/kg/hr. at 3 cc/min.							
2:10-2:20	4.5	63.51	0.15	5.13	0.81	10.3	63.51	1.67
2:20-2:30	3.4	56.81	0.15	5.47	0.96	11.1	56.81	1.84
2.35	Nicotinamide Priming dose 2.0 mg/kg. i.v.							
	Venoclysis 2.0 mg/kg/min. (added to the NMN venoclysis)							
	NMN Venoclysis continued as above							
2:50-3:00	2.3	46.11	70.0	3.47	.75	17.6	46.11	1.70
3:00-3:10	2.2	48.73	76.0	3.99	.82	23.4	48.73	1.44

TABLE 8. LACK OF EFFECT OF TRIGONELLINE AND 1-METHYL-3-CARBOXYLAMIDE-6-PYRIDONE ON RENAL CLEARANCE OF NMN

URINE FLOW	CREATININE CLEARANCE	N ¹ -METHYLNICOTINAMIDE		
		Plasma Conc.	Clearance	Clearance ratio
<i>cc/min.</i>	<i>cc/min.</i>	<i>γ/cc.</i>	<i>cc/min.</i>	
<i>Trigonelline¹ Experiment</i>				
Control phase: NMN values, Dog 365 wt. = 13.6 kg.				
3.5	55.0	10.9	92.6	1.68
3.6	59.2	11.7	105.8	1.78
Trigonelline: Priming dose 100 mg/kg. i.v.				
Venoclysis 125 mg/kg/hr. i.v.				
4.5	52.4	12.2	111.8	2.13
4.5	61.4	12.8	132.9	2.16
<i>1-Methyl-3-carboxylamide-6-pyridone² Experiment</i>				
Control phase: NMN values, Dog 84 wt. = 17.1 kg.				
2.7	59.49	27.88	88.60	1.44
2.7	57.80	32.29	82.27	1.42
Methylcarboxylamidepyridone: Priming dose 12.5 mg/kg. i.v.				
Venoclysis 15 mg/kg/hr. i.v.				
3.0	62.23	37.66	99.07	1.59
3.4	60.57	45.89	84.12	1.39

¹ NMN Priming dose 0.25 mg/kg. i.v. Venoclysis 0.25 mg/kg/hr. in 5% glucose at 3 cc/min. throughout the experiment.² NMN Priming dose 0.5 mg/kg. i.v. Venoclysis 0.5 mg/kg/hr. at 3 cc/min. throughout the experiment.

concentrations should have been adequate for this purpose if such an effect were demonstrable. Actually, trigonelline is not formed in the body (15), is quite non-toxic (acute intravenous LD₅₀ for mice = 2300 ± 360 mg/kg.), and presents no problem for elimination by the body in the amounts ordinarily ingested. Since 1-methyl-3-carboxylamide-6-pyridone does not contain a quaternary ammonium ion, its lack of an inhibitory effect on NMN excretion is consistent with the rest of the data. This 6-pyridone has been isolated from human urine as a metabolite of nicotinamide (26).

If one compares the toxicity of nicotinic acid, nicotinamide, and N¹-methylnicotinamide with what is known about their renal elimination, the results are of considerable consequence to one interested in the way the mammalian body disposes of compounds. In mice the acute intravenous toxicity of nicotinic acid is 1170 ± 143 mg/kg. As this is converted into the amide in the body the toxicity is reduced still further, the corresponding i.v. LD₅₀ of nicotinamide being 2100 ± 155 mg/kg. Since this amide is an essential metabolite, it is consistent that its renal elimination should be very low; i.e. that its reabsorption or conservation by the kidneys should be great. It seems almost paradoxical that in the inactivation of nicotinamide by transmethylation the body should produce a compound (NMN) having an intravenous acute toxicity of 345 ± 26 mg/kg. (mouse). This transmethylation, then, results in more than a six-fold increase in toxicity. Thus, the rapid elimination of NMN by tubular secretion in addition to glomerular filtration would seem to be a compensatory measure for the increased toxicity of this nonessential metabolite of nicotinamide. Except in the dog, NMN excretion represents a fairly small percentage of the total dose of nicotinamide administered (27). By far the greater amount of nicotinamide metabolite is believed to be a pyridone or other degradation product. Thus it seems that in the metabolism of nicotinamide the body makes use of both renal tubular secretion of toxic products plus, in some animals, a further oxidation of the amide to less toxic products whose rates of renal elimination are of less vital significance, ordinarily. Consistent with this interpretation is the observation that the acute intravenous LD₅₀ of the 6-pyridone in mice is 1310 ± 80 mg/kg., or roughly one fourth that of its probable precursor (NMN).

CONCLUSIONS

N¹-methylnicotinamide (NMN) is excreted by the combined processes of glomerular filtration and tubular secretion. The transport mechanism for the tubular secretion of NMN is distinct, functionally, from that responsible for the secretion of p-aminohippurate, penicillin, and phenol red by the tubules.

Carinamide, which blocks the tubular secretion of p-aminohippurate and compounds eliminated by that same tubular mechanism, does not interfere with the tubular transport system for NMN secretion. The compounds secreted by the two cellular mechanisms are not mutually competitive, nor do they suppress the functional capacity of the opposite transport system, within the limits of these experiments. NMN does not appear to be bound on plasma components according to our measurements.

Nicotinamide, trigonelline and 1-methyl-3-carboxylamide-6-pyridone do not inhibit the renal elimination of N¹-methylnicotinamide.

It is a pleasure to acknowledge the cooperation of our associates that helped to make this study possible. Dr. J. W. Huff supplied the nicotinic acid derivatives and counceled the setting up of the N¹-methylnicotinamide method, of which he is co-author. Dr. L. D. Wright supervised the estimation of nicotinamide by the method of which he is co-author. Dr. Harold M. Peck participated in the simultaneous renal extraction and clearance experiments, of which a more detailed presentation of the methodology has been presented by him elsewhere (28). Mr. S. E. McKinney and his associates, Mrs. B. J. Angstadt and Mr. J. M. Bochey, were responsible for the preliminary toxicity data reported herein.

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CHANGES IN RENAL FUNCTIONS ASSOCIATED WITH DIABETES INSIPIDUS PRECIPITATED BY ANTERIOR HYPOTHALAMIC LESIONS¹

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A NUMBER of observations indicate that the hypophysis exercises a prominent influence on renal functions. Removal of the anterior lobe or both lobes of the hypophysis in rats tends to inhibit water diuresis (1). Removal of the anterior lobe of the hypophysis in dogs profoundly reduces the inulin and diodrast clearances and the diodrast Tm (2). However, it has been reported that no changes occurred in renal function (2) in two dogs with diabetes insipidus produced by hypothalamic lesions.

This report concerns the changes in glomerular filtration, renal plasma flow and the renal tubular transport maxima for dextrose that occur in diabetes insipidus, produced by a large bilateral, anterior hypothalamic lesion in the dog.

SURGICAL PROCEDURES

Diabetes insipidus was precipitated in dogs 5, 7, 8, 19 and 16 Z by uncomplicated bilateral, anterior hypothalamic puncture and in 17, 20, 22 and 26 Z by complete high scissors-section of the hypophysial stalk close to its hypothalamic attachment followed by maceration of the ventral, anterior hypothalamus (anterior hypothalamic puncture). Both procedures were carried out by the subtemporal surgical approach to the hypophysis.

The photographs shown in figure 1 illustrate the microscopic appearance of the region involved in the uncomplicated hypothalamic puncture procedure. We wish particularly to stress the complete absence of anatomical or functional disturbance of the adenohypophysis. The animals post operatively exhibited no signs of hypopituitary function. They uniformly reacted normally to insulin and environmental tolerance tests. At autopsy there was no evidence of adrenal cortical atrophy.

METHODS

Creatinine was used to measure glomerular filtration rate (GFR), p-amino-hippurate for renal plasma flow (RPF) and dextrose for the maximum reabsorptive capacity of the renal tubules (TmG). The technique and methods used for determin-

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ing the above substances were as previously described (3). No anesthesia or other medication was given during the measurement of renal functions. All these dogs under study had permanent diabetes insipidus and were eliminating 2500 to 6000 cc. of urine per day when maintained on a constant daily and individually equivalent food intake. They were observed over a period of many months following the operation.

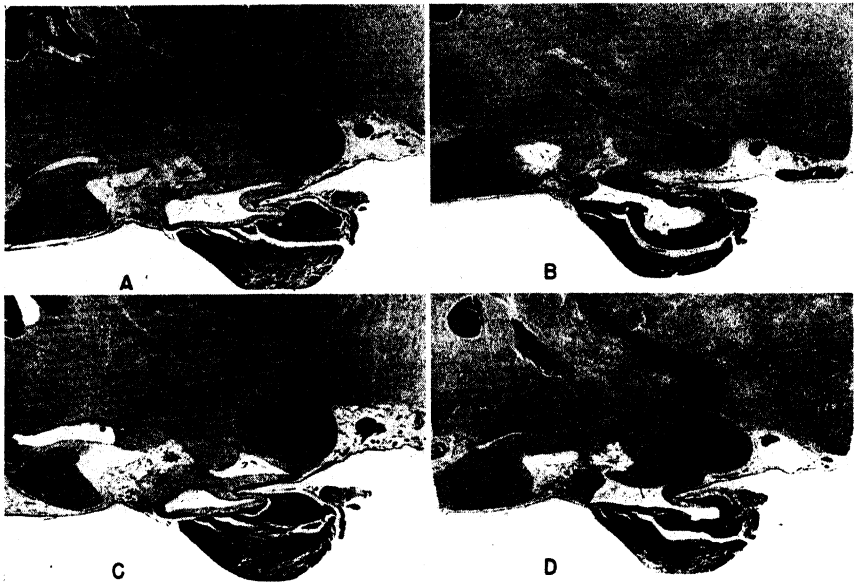


Fig. 1. REPRESENTATIVE SAGITTAL SECTIONS taken from an *H* and *E* series on dog 10 Z. Sections *A* and *B* are from the left of the mid-line; *C* and *D* are equivalent sections from the right side. The photomicrographs demonstrate that: 1) the entire adenohypophysis is undisturbed and even the anterior strip of pars tuberalis is in its normal location and completely intact; 2) the anterior infundibulum is markedly thinned and the infundibular process is absent, having sloughed completely; 3) the ventral portion of the anterior hypothalamus is destroyed, including the caudal aspect of the optic chiasm; 4) the posterior hypothalamus and posterior infundibulum (tuber cinereum) are undisturbed.

RESULTS AND DISCUSSION

From table 1, it is apparent that drastic reductions in the creatinine clearance, renal plasma flow and reabsorptive capacity of the renal tubules for dextrose have occurred, reductions that in most of the animals were greater than 50 per cent. These changes occurred within 10 days to 2 weeks after the operation, but no attempt was made to determine the exact time of onset.

The reduction in glomerular filtration, renal plasma flow and TmG seems to be permanent phenomena associated with diabetes insipidus produced in this manner. These effects have been found to persist for more than 2 years following the operation. Since the changes in renal plasma flow and the TmG are approximately proportional to the reduction in glomerular filtration rate, we conclude that these changes are due

to a decrease in the number of active nephrons in the kidneys of these animals. It is conceivable that these effects on the kidney are a manifestation of a protective mechanism for conserving body water, since, presumably, the rate of water loss would be greater if glomerular filtration were maximal. Experiments on normal dogs have shown that it is possible to produce similar changes in renal hemodynamics by moderate dehydration (3). The changes in kidney functions are not the result of

TABLE 1. CHANGES IN RENAL FUNCTION AFTER DEVELOPMENT OF PERMANENT DIABETES INSIPIDUS¹

DOG NO.	WT.	GFR	RPF	TmG	$\frac{GF}{TmG}$	FF
	kg.	cc/min.	cc/min.	mg/min.		
5-Z	9.5	48	153	168	0.29	0.26
		23	75	68	0.33	0.31
7-	11.5	62	250	223	0.28	0.25
		28	116	103	0.27	0.24
8-	11.0	55	173	190	0.29	0.31
		22	67	85	0.26	0.33
10-	9.5	40	121	129	0.31	0.33
		26	70	91	0.29	0.37
16-	13.0	48	134	171	0.28	0.36
		19	66	64	0.30	0.29
17-	10.0	44	191	160	0.27	0.23
		27	84	112	0.24	0.32
20-	14.0	60	221	200	0.30	0.27
		21	60	110	0.35	0.29
22-	11.0	51	152	186	0.27	0.34
		20	55	83	0.24	0.36
26-	17.0	65	223	232	0.29	0.28
		31	142	120	0.22	0.26

¹ The initial set of figures in the table for each dog represents an average of several control determinations before operation. The second row of figures is an average of several determinations made after the development of diabetes insipidus.

permanent renal damage. We have found it possible to restore glomerular filtration and the other renal functions to the preoperative level by infusing saline.

This study demonstrates that the destruction of the neurohypophysis results in prominent changes in kidney function besides an impairment in the capacity of the renal tubules to reabsorb water. Moreover, these effects occur in the absence of any significant anatomical or functional damage to the adenohypophysis (fig. 1). This suggests that the changes in renal functions seen in these animals are neurogenic in origin rather than due to a disturbance in the endocrine system, as has been suggested for similar changes seen in hypophysectomized dogs (4).

SUMMARY

With the development of diabetes insipidus following the anatomical destruction of the neurohypophysis without damage to the adenohypophysis, glomerular filtration rate, renal plasma flow and the maximal rate of transfer of dextrose, all decrease

50 per cent or more. These changes are proportional and are believed to be caused by the inactivation of nephrons with the development of diabetes insipidus. This may be an additional mechanism for conserving water, since it seems to operate in normal animals subjected to moderate dehydration.

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RENAL CLEARANCE OF FERROCYANIDE IN THE DOG

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THE ferrocyanide ion was one of the earlier substances suggested as a measure of the rate of formation of glomerular filtrate. The possibility that ferrocyanide might be excreted by filtration only was suggested by histochemical studies which showed it to be absent from the tubular cells when present in high concentration in plasma and the tubular lumen (1) and by its absence from the urine of aglomerular fish (2). A study of the clearances of ferrocyanide, inulin and creatinine (3) led Van Slyke, Hiller and Miller to conclude that ferrocyanide was, indeed, suitable for the measurement of filtration rate in the dog and rabbit. The data on which these conclusions were based (mean ferrocyanide-creatinine ratio 0.96, $\sigma = 0.18$ in 27 comparisons), while showing no significant differences among the clearances of these substances, were not sufficiently uniform to establish their identity. Little, if any, additional data on ferrocyanide clearances in animals have since been published. Doubt as to the usefulness of ferrocyanide in renal physiological studies was introduced by the study of Miller and Winkler (4) in man. These authors not only found the clearance of ferrocyanide in man to be of the order of magnitude of that of urea, suggesting appreciable reabsorption, but reported evidences of considerable nephrotoxicity.¹

The results of a re-examination of the renal clearance of ferrocyanide indicate that ferrocyanide is probably neither reabsorbed nor secreted by the renal tubules of the dog.

MATERIALS AND METHODS

Studies were performed on trained, normal female dogs weighing 12 to 20 kg. Creatinine to yield plasma concentrations of about 20 mg. per cent and ferrocyanide were administered by continuous intravenous infusion. Urines were collected through an indwelling catheter and each clearance period terminated by washing the bladder with distilled water. Heparinized arterial blood samples were obtained at the midpoint of each clearance period.

Ferrocyanide in blood and urine was determined by a method which utilizes the spectral absorption peak of ferricyanide at a wave length of 420μ as follows: *Plasma.* To 2 ml. of plasma add 10 ml. of distilled water and 8 ml. of 10 per cent vacuum-distilled² trichloroacetic acid. *Filter.* To duplicate 5 ml. aliquots of filtrate

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¹ Dr. Kendrick Hare (5) informs us that in a number of observations he has found the ferrocyanide and creatinine clearances identical in the dog.

² C.P. trichloroacetic acid contains sufficient amounts of iron to interfere with the determination. No other protein precipitant was found satisfactory since the heavy metal salts of ferrocyanide are insoluble and tungstic acid itself gives an appreciable light absorption at 420μ .

add 0.4 ml. of 1.5 per cent hydrogen peroxide. Allow to stand 15 min. Read in photoelectric colorimeter at 420 μ .

Urine. Dilute urine to the concentration expected in the plasma filtrate. To duplicate 5 ml. aliquots add 0.2 ml. 12 N HCl and 0.2 ml. 3 per cent H_2O_2 . Allow to stand 15 minutes. Read at 420 μ .

The concentrations are read from a standard curve prepared by the procedure used for urine. Light absorption by the ferricyanide does not follow Beer's Law. Since optical density is not directly proportional to concentration, multiple points are desirable covering the range of concentrations to be determined. A range of final concentrations from 0.2 to 0.6 $\mu M/l.$ is satisfactory when readings are made in 5 ml. cuvettes on the Coleman, Jr. spectrophotometer (model 6A).

Thirty-eight plasma recoveries corresponding to plasma concentrations of 2 to 6 mm/l. gave an average recovery of 98.5 per cent with a range from 94 to 103 per cent, half of the recoveries falling between 99 and 101 per cent. Plasma blanks are

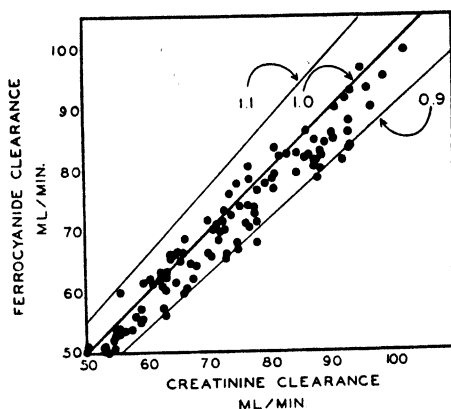


Fig. 1. RELATIONSHIP of ferrocyanide to creatinine clearance. Mass plot of all clearance comparisons. The diagonal lines represent the indicated ratios of ferrocyanide to creatinine clearance.

negligibly small, having an optical density of not more than 0.009 when read in 5 ml. cuvettes on the Coleman, Jr., spectrophotometer.

Creatinine in tungstate filtrates of plasma and in diluted urine was determined by a modification of Folin's method (6).

RESULTS

The clearance of ferrocyanide was compared with that of creatinine in 16 experiments on 8 dogs, yielding a total of 109 clearance periods for comparison. The ferrocyanide-creatinine clearance ratios for individual clearance periods were normally distributed about a mean of 0.966 with a standard deviation of 0.041. The relationship of ferrocyanide clearance to creatinine clearance is shown in figure 1. There was no tendency for individual dogs to show consistently high or low clearance ratios. No toxic effects due to ferrocyanide were observed in any experiment.

DISCUSSION

Ferrocyanide, being a tetravalent anion, must, according to the Gibbs-Donnan law, appear in the glomerular filtrate at slightly higher concentration than in the

plasma water. The expected ratio is the fourth root of the ratio for chloride or about 1.01. This is also, then, the ratio of ferrocyanide to creatinine clearance which would be expected if no ferrocyanide were secreted or reabsorbed. Although the mean ratio found, 0.966, differs only slightly from the theoretical of 1.01, the difference is statistically significant. Such a difference could be due to binding of ferrocyanide to plasma proteins, to reabsorption of a very small amount of ferrocyanide, or to a small, undetected systematic error in the determination of the clearance of either

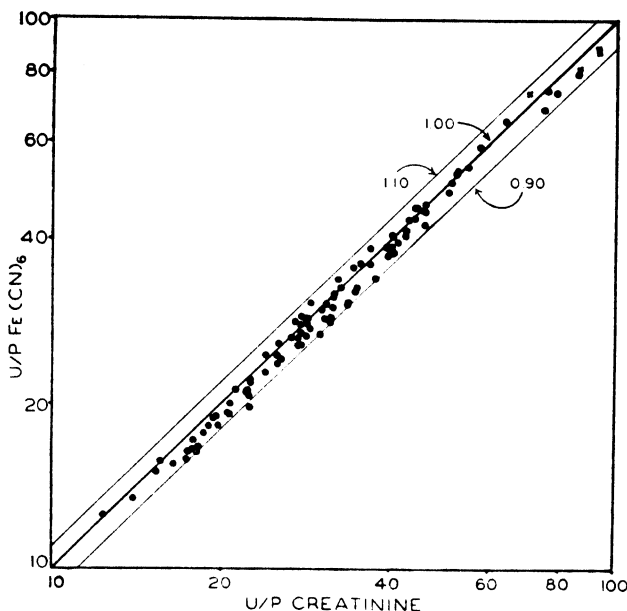


Fig. 2. RELATIONSHIP of ferrocyanide U/P ratio to creatinine U/P ratio. Plot, on logarithmic scale, of all clearance comparisons. Points indicated X have been plotted at 10 times the observed value. Diagonal lines indicate ratios of ferrocyanide to creatinine clearance.

substance. Dialysis of ferrocyanide-containing plasma through cellophane membranes against isotonic phosphate buffer yielded no evidence of binding on plasma proteins.

If ferrocyanide were reabsorbed by passive diffusion, the extent of reabsorption should be related to the degree of concentration of the urine. This does not appear to be the case as shown by the data in figure 2. The U/P ratio of ferrocyanide has been plotted against the U/P ratio for creatinine on a logarithmic scale. The relationship of the two concentration ratios shows no trend with respect to the degree of urinary concentration. Were the ferrocyanide passively reabsorbed, lower ferrocyanide-creatinine ratios would be expected at higher U/P ratios.

A plot of the ferrocyanide-creatinine clearance ratios against the plasma ferrocyanide concentration shows no relationship (fig. 3). A tendency for the ratio to increase with increasing plasma concentration would suggest active reabsorption. The absence of such a relationship, while not conclusive because of the relatively

limited range of plasma concentrations studied, may be considered evidence against the hypothesis of active tubular reabsorption. The administration of mercurial and osmotic diuretics had no effect on the clearance ratio.

Since in the method described all ferrocyanide is converted to ferricyanide before reading, the material determined is actually the sum of ferrocyanide plus ferricyanide. No measurable amount of ferricyanide (as estimated from the colorimeter reading before oxidation with peroxide) could be found in either plasma or urine during the administration of ferrocyanide nor in the arterial blood during the intravenous administration of ferricyanide. However, since ferricyanide added to blood *in vitro* is rapidly converted to ferrocyanide, absence of ferricyanide from the blood *in vivo* could not be established. The clearance of apparent ferrocyanide was not altered by the replacement of the infused ferrocyanide with ferricyanide. It is probable that ferricyanide is rapidly converted to ferrocyanide in the body so that the form in which it is administered is not of major importance. There is a possibility that a small amount of the anion is present in oxidized form and that this

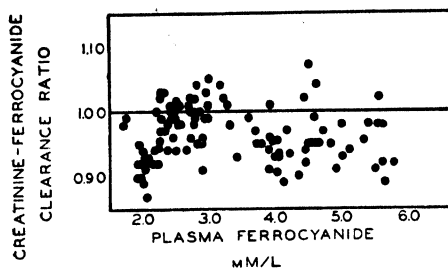


Fig. 3. RELATIONSHIP between ferrocyanide-creatinine clearance ratio and plasma ferrocyanide concentration.

species is reabsorbed by the renal tubules. This seems unlikely and would be most difficult to establish.

The technical procedures for the determination of the clearances have been reviewed and no source of systematic error has been detected. However, this would still appear to be the most likely explanation of the minimal difference between the clearance of ferrocyanide and that of creatinine. The difference is so small that to interpret it as attributable to anything other than experimental error does not seem warranted. With slight reservation pending explanation of the observed difference, it seems reasonable to conclude that ferrocyanide is excreted at the level of glomerular filtration.

SUMMARY

A simple colorimetric method for the determination of ferrocyanide is described. A comparison of the clearance of ferrocyanide and creatinine gave an average clearance ratio of 0.966 with a standard deviation of 0.41 in 109 observations. The very slight difference found is most probably attributable to systematic experimental error and it is concluded that ferrocyanide is excreted at the level of glomerular filtration.

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CITRIC ACID AND ITS RELATION TO SERUM AND URINARY CALCIUM

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THE citric acid content of human blood varies from 1.5 to 4.0 mg. per cent (1), whereas the whole blood of dogs contains 0.9 to 1.9 mg. per cent (2). Values for the normal daily excretion of citric acid by human adults vary from 0.2 to 1.0 gm. daily (3). The daily excretion in the urine of adult dogs is much less.

It has been reported that the citric acid level in the blood and urine may be influenced by age (4), sex (5), muscular work (6), diet (7, 8, 9), administration of citrates (9, 10, 11) and citrate precursors¹ (10, 12), and by parathormone (13) and sex hormones (14). The urinary pH is also a factor affecting the excretion of citric acid (15).

Dickens (16) was the first to demonstrate that most of the citric acid in the body is concentrated in the skeleton, presumably as the calcium salt. Others (17, 18) have also identified it in calcium concretions found in the body. Citrate has been shown (19-29) to form a soluble complex with calcium ions. This property enables citrate ions to dissolve insoluble calcium deposits and has led to the practical use of a buffered citrate solution as a lavage to remove urinary tract calculi (30).

Gomori and Gulyas (31) found that subcutaneous injections of citrate (8 to 30 cc. of 4% sodium citrate/kg. body weight) into dogs cause a considerable and prompt increase in the urinary excretion of calcium without affecting the blood calcium. These workers found that repeated subcutaneous injections of citrate into puppies and rats lead to osseous changes very similar to those produced by large doses of parathormone. Alwall (13) gave parathormone to dogs and observed a parallel rise in serum citric acid and calcium. Shorr and co-workers (32) reported parallel changes in the urinary excretion of calcium and citric acid by a hypoparathyroid patient who received injections of parathormone. Calcium injections produced similar results in another patient with hyperparathyroidism.

The physical and chemical properties of calcium and citric acid are such that alterations in the concentration of either substance in body fluids are likely to influence the concentration and state of the other substance. The extent to which either substance plays a role in defining the normal metabolic characteristics of the other substance remains to be established. It is also not clear whether or not a quantitative relation exists between the concentration of citric acid and calcium in blood and urine. The present study was undertaken to determine to what extent the injection of either calcium or citric acid influences the blood and urinary concentration of the other substance. It was necessary to exclude effects on the acid-base balance as a possible explanation of some observed changes.

PROCEDURE

Six healthy adult female dogs were used for this study. They were maintained on a diet of constant composition. All animals were fasted 15 to 24 hours prior to an experiment.

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¹ Lactate, malate, fumarate, succinate, etc.

The following solutions were injected in this study: 1) 2.5 per cent citric acid neutral to a pH 7.4 with sodium hydroxide just before injection; 2) 2.5 per cent solution of anhydrous calcium chloride; 3) 2.5 per cent solution of ammonium chloride; 4) 3.3 per cent solution of sodium bicarbonate, and 5) 5 per cent solution of monocalcium citrate.²

With 2 exceptions all solutions were given at a rate of 40 mg/kg/hour for 2 hours. These exceptions were sodium bicarbonate, given at a rate of 55 mg/kg/hour, and calcium citrate, given at a rate of 80 mg/kg/hour. In every experiment 0.85 per cent sodium chloride was injected during a control period of one hour at the same rate of injection as was to be used during the following experimental period. The animals were secured on a table during the entire experiment. Solutions were injected dropwise into a limb vein by means of a gravity drip apparatus.

Venous blood samples were secured from the external jugular vein before and at hourly intervals during the injection period. The 24-hour urine samples were collected in clean metabolic cages using 10 cc. of 10 N sulphuric acid as a preservative. The urine samples during injection of a solution were collected by catheterization at intervals corresponding to those when blood samples were taken. For the sake of comparison fasting blood samples and 24-hour urine samples were determined. The 24-hour urine specimens were completed by catheterization.

Plasma and urinary citric acid were estimated according to the method of Pucher, Sherman and Vickery (2), using the sodium sulfide reagent of Hunter and Leloir (33) and read in a Coleman photoelectric colorimeter with filter No. 42. Serum calcium was determined by the method of Kramer and Tisdall (34) as modified by Clark and Collip (35), using Wang's wash (36) and further modified according to the practice in this laboratory of using round bottomed centrifuge tubes and 4-hour precipitation of the calcium oxalate. All estimations were performed in duplicate and agree within 5 per cent or less.

RESULTS

The normal plasma citric acid, serum calcium, urinary citric acid and urinary calcium of the 6 female dogs are shown in table 1. The values for each animal are the average of several determinations at different times. The level of citric acid varies considerably from animal to animal, whereas the serum calcium content is uniform in all 6 animals.

In tables 2 and 3 are shown the effects of various solutions injected intravenously on the average plasma citric acid and serum calcium and urinary citrate and calcium of these same animals. The injection of citrate markedly increased its concentration in blood and urine. At the same time there was a rise in the serum and urinary concentration of calcium. The rise in the rate of urinary excretion of calcium was more striking than the elevation in serum calcium which resulted from citrate injection.

The rate of calcium and of citrate excretion during the second hour of injection was approximately twice that during the first hour of injection. Changes in blood concentration during this time were relatively slight. The data in tables 2 and 3 indicate that a rise in plasma citric acid increased the serum calcium value

² The monocalcium citrate used in this study was provided by Dr. Lathan C. Crandall, Jr., Ames Laboratories, Elkhart, Indiana.

and that an increase in urinary citrate output is accompanied by a higher urinary excretion of calcium. The injection of normal saline did not produce any appreciable effects on either the citric acid or calcium levels in blood or urine.

TABLE 1. BLOOD AND URINE CITRIC ACID AND CALCIUM OF 6 NORMAL FEMALE DOGS

DOG NO.	BODY WT.	PLASMA CITRIC ACID	SERUM Ca	URINARY CITRIC ACID	URINARY Ca
	kg.	mg. %	mg. %	mg/hr.	mg/hr.
364	10	1.50	10.40	0.33	0.38
366	12	3.38	11.34	0.24	1.10
367	12.5	3.37	10.69	0.76	1.33
368	12.5	2.67	11.20	0.40	0.85
371	13.0	3.11	10.10	0.66	1.05
383	15.5	4.18	11.46	0.72	1.72
Average.....	12.6	3.04	10.87	0.52	1.07

TABLE 2. AVERAGE VALUES OF PLASMA CITRIC ACID AND SERUM CALCIUM PRODUCED BY INJECTIONS IN NORMAL DOGS

NO. OF DOGS	COMPOUND INJECTED FOR 2 HOURS	PLASMA CITRIC ACID			SERUM CALCIUM		
		Control	1st. hr.	2nd hr.	Control	1st hr.	2nd hr.
	mg/kg.	mg. %			mg. %		
6	Citric acid (80)	3.38	18.42	21.33	10.87	11.41	11.80
6	CaCl ₂ (Cl = 51) (Ca = 29)	3.31	3.60	3.21	10.92	14.72	16.13
6	NH ₄ Cl (Cl = 53)	3.12	3.52	3.42	10.83	10.81	11.21
3	NaHCO ₃ (Na = 100)	5.05	5.26	4.66	11.38	11.29	11.20
6	Ca-Cit. (Cit. = 132) (Ca = 28)	4.71	30.63	34.55	10.89	15.24	16.45

TABLE 3. AVERAGE VALUES OF URINARY CITRIC ACID AND CALCIUM PRODUCED BY INJECTIONS IN NORMAL DOGS

NO. OF DOGS	COMPOUND INJECTED	URINARY CITRIC ACID			URINARY Ca		
		Control	1st hr.	2nd hr.	Control	1st hr.	2nd hr.
		mg/hr.			mg/hr.		
6	Citric acid	0.54	41.07	91.80	0.39	4.60	8.91
6	CaCl ₂	0.46	0.16	0	0.64	2.23	2.43
6	NH ₄ Cl	0.50	0.47	0.49	0.57	0.63	0.91
3	NaHCO ₃	1.22	1.46	1.58	0.59	0.87	1.49
6	Ca-Cit.	1.16	187.0	361.27	0.59	15.90	44.08

It remained to be determined whether or not calcium injections would influence the citrate content of blood and urine. The same dogs were used for these experiments. From the results in tables 2 and 3 it is apparent that calcium injections were without significant effect on the blood citric acid level despite the fact that the serum calcium values reached levels at which the pharmacological effects of calcium become

apparent. The urinary output of citric acid actually decreased after calcium chloride injection, although there was a significant increase in the rate of calcium elimination. From these observations it seems apparent that calcium does not increase the citric acid in blood and urine as the latter did the former. The decrease in citrate excretion produced by calcium chloride injection is definite.

Ammonium chloride and sodium bicarbonate were injected to ascertain to what extent alterations in acid-base balance might influence blood and urine levels of citric acid and calcium. From the data in tables 2 and 3 it is apparent that neither substance significantly altered the blood concentration of citric acid or calcium. Both substances produced some increase in the rate of calcium excretion in the urine. Ammonium chloride did not influence the citric acid content of urine, but sodium bicarbonate injection caused a slight augmentation of citric acid excretion in the urine. The lack of effect of ammonium chloride injections on the citrate content of urine indicates that the decreased citrate excretion produced by the injection of calcium chloride must have been due to calcium. The slight but definite increase in calcium excretion caused by ammonium chloride must be explained by some mechanism other than that involving a citrate effect on calcium excretion. The slight increase in calcium excretion associated with sodium bicarbonate injection may have been due either to formation of calcium bicarbonate or to the increased citrate excretion or to both. Two of the 3 dogs receiving sodium bicarbonate injections were in heat, which probably accounts for the higher control values for calcium and citric acid on these animals.

Monocalcium citrate was also injected to compare the effects of this compound on the blood concentrations of calcium and citric acid as compared to either substance injected alone. Calcium citrate was injected so that the calcium injection was essentially the same as when calcium chloride was administered. The greater elevation of blood citrate after calcium citrate injection was what one might expect with the higher dosage of citrate/kg/hour. The elevation of serum calcium was essentially the same as that which occurred when the highly ionized calcium chloride was injected. There was a much greater loss of calcium and of citrate in the urine following injection of calcium citrate than following injection of either sodium citrate or calcium chloride.

The greater urinary calcium loss at essentially the same serum value as existed during CaCl_2 administration may have been due to the greater filterability of the serum calcium following calcium citrate injection or because of decreased reabsorption from the glomerular filtrate of the calcium citrate complex. It is apparent from these experiments that the state of calcium in the blood stream greatly influenced its urinary excretion.

SUMMARY

Injection of citric acid neutralized with sodium hydroxide to pH 7.4 increased the serum calcium value and the urinary excretion of calcium. Injection of calcium chloride did not alter the citric acid content of blood plasma but reduced the urinary excretion of citric acid. At the same time the calcium output in the urine increased.

The above-mentioned effects were not caused by changes in acid-base balance,

since comparable amounts of sodium bicarbonate and ammonium chloride did not produce comparable effects. Injection of monocalcium citrate produced a similar serum calcium elevation but a much greater urinary calcium excretion than the same amount of calcium injected as calcium chloride.

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ROLE OF THE KIDNEY AND OF CITRIC ACID IN PRODUCTION OF A TRANSIENT HYPERCALCEMIA FOLLOWING NEPHRECTOMY

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IT IS recognized that the kidneys and parathyroid glands are intimately related in functions that pertain to the metabolism of calcium and phosphorus (1-6). Patients with advanced renal insufficiency frequently have hyperplasia of the parathyroid glands associated with a low serum calcium and a high content of inorganic phosphorus in the blood.

Experiments of Martensson (7) showed that the serum citric acid of the nephrectomized rabbit rises rapidly up to four to five times its normal value. By perfusion of isolated cat kidneys with heparinized blood, the same author found that the renal parenchyma oxidized citric acid very actively. Gomori and Grulyas (8) observed that injecting citric acid into dogs increased the urinary excretion of calcium, left the serum calcium unchanged, and did not produce definite changes in phosphorus excretion. The serum calcium became more filterable as a result of citrate injections. The *in vitro* experiments of Kuyper (9) indicate that the precipitate formed in the presence of suitable concentrations of calcium, phosphate and citrate was actually a complex of the 3 such as may exist in bone. In blood there is probably a calcium-citrate complex present (10-16). This complex is soluble, diffusible and largely unionized. A rise in the citrate content of blood may reduce the calcium ion of muscle (17), influence oxidation systems (18, 19) and cause convulsions (13).

In the preceding paper it was shown that an increase in the citrate content of blood and urine will lead to a rise in the serum and urinary calcium values. The present report is concerned with the extent to which the kidney may influence the calcium and citrate content of the blood. In this connection the role of the kidney in the destruction of injected citrate was also studied.

METHODS

Bilateral nephrectomy was carried out on 12 healthy adult dogs. Six of these animals were injected with partially neutralized citric acid (40 mg/kg.) 48 hours after nephrectomy. Bilateral ligation of the ureters was performed on 6 healthy adult dogs. All of these animals received injections of citrate. All operations were performed aseptically and no infectious processes were observed at autopsy. Water was allowed *ad libitum* after the operation. All animals were fasted 18 to 24 hours prior to the operation.

Injections and analytical procedures for citrate and calcium are as indicated in the preceding paper. Serum inorganic phosphorus was estimated by the Gomori (20) modification of the Kuttner and Lichtenstein method (21). Non-protein nitrogen was determined according to the method of Koch and McMeekin (22).

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RESULTS

Effect of Bilateral Nephrectomy. Bilateral nephrectomy was done on 3 dogs and the changes in the blood citric acid, calcium, inorganic phosphorus and non-protein nitrogen were followed every 24 hours until death. The average results of these 3 dogs are shown in table 1. There is a marked elevation in plasma citric acid within 24 hours after the operation (4.03 mg. % to 19.50 mg. %). After this initial rise there was a rapid drop in the next 24 hours and it remained somewhat lower than normal until death, although there was a slight terminal rise.

TABLE 1. EFFECT OF NEPHRECTOMY ON SERUM VALUES OF ADULT DOGS

POSTOPERATIVE HOURS	CITRIC ACID	CALCIUM	INORGANIC PHOSPHORUS	NON-PROTEIN NITROGEN
	mg. %	mg. %	mg. %	mg. %
Initial	4.03	10.39	3.59	48.1
24	19.50	12.50	9.71	102.9
48	4.50	11.28	12.86	185.5
72	3.00	10.18	21.18	265.3
96	3.90	8.43	31.33	373.9

TABLE 2. EFFECT OF CITRIC ACID INJECTION UPON SERUM VALUES OF NEPHRECTOMIZED DOGS

POST- OPERATIVE HOURS	NO. OF DOGS	CITRIC ACID	CALCIUM	PHOS- PHORUS	NON- PROTEIN NITROGEN	POST- OPERATIVE HOURS	NO. OF DOGS	CITRIC ACID	CAL- CIUM	PHOS- PHORUS	NON- PROTEIN NITRO- GEN
		mg. %	mg. %	mg. %	mg. %			mg. %	mg. %	mg. %	mg. %
Initial	6	3.45	10.77	3.56	41.0	52 ³	6	46.90	13.58	9.79	184.3
24	6	31.69	13.41	8.91	91.0	72	6	32.60	11.44	16.36	194.8
48	6	23.06	12.00	10.53	164.9	96	5	35.00	9.97	22.64	235.4
40 ¹	6	61.60	13.02	9.38	159.1	120	3	42.20	9.96	29.82	345.4
50 ²	6	54.30	13.46	9.94	165.1	Terminal	5	25.40	8.36	36.70	365.6

¹ Immediately after injection. ² One hour after injection. ³ Three hours after injection.

The changes in serum calcium for the most part paralleled those of citric acid. The calcium increased during the first 24 hours after nephrectomy from 10.39 to 12.50 mg. per cent and returned to the normal range in the next 48 hours. If the animals survived longer than 72 hours the serum calcium usually fell to values below normal. The increase in serum inorganic phosphorus and non-protein nitrogen was continuous and usually paralleled one another. The average survival time for these 3 animals was 98.3 hours.

Effect of Injection of Citric Acid on Bilaterally Nephrectomized Dogs. The blood constituents under consideration were determined before and at regular intervals after nephrectomy as shown in table 2. Forty mg/kg. of citric acid (2.5% solution neutralized to pH 7.4 with sodium hydroxide) was injected into 6 nephrectomized dogs. Control injections of saline as well as the citric acid injections were given for one hour. Citric acid was injected 48 hours after nephrectomy. As in the previous 3

animals, removal of the kidneys caused a rise in plasma citric acid and in the serum calcium values. The average rise in citric acid 24 hours after nephrectomy was from 3.45 to 31.69 mg. per cent. At the end of 48 hours the citric acid and calcium values were still well above their control concentrations.

The response of nephrectomized dogs to the injection of citric acid is qualitatively the same as that of the normal animal. The blood concentration of both substances definitely increased in every instance. Three hours after injection of citrate the average plasma concentration of citric acid had increased from 23.06 mg. per cent to 46.90 mg. per cent, whereas the serum calcium level had increased from 12.00 to 13.88 mg. per cent. There was a gradual decline in the plasma citric acid level during the subsequent days of survival. The plasma level of citric acid remains well above the normal range and it is apparent that the nephrectomized dog has a limited ability to destroy intravenously injected citric acid. There is a gradual decline in the serum calcium concentration during the remainder of the survival period. This decline is less striking than that usually observed in nephrectomized dogs with such a

TABLE 3. AVERAGE SERUM VALUES OF 12 DOGS BEFORE AND 24 HOURS AFTER NEPHRECTOMY

	NO. OF DOGS	CITRIC ACID		CALCIUM		INORGANIC PHOSPHORUS		NON-PROTEIN NITROGEN	
		mg. %		mg. %		mg. %		mg. %	
		Range	Average	Range	Average	Range	Average	Range	Average
Initial	12	2.65-5.60	3.98	9.31-11.87	10.58	2.45-5.04	3.88	30.3-59.1	43.8
24 hours after operation	12	5.65-42.7	21.66	10.67-14.79	12.97	7.10-13.9	9.67	79.5-127.0	100.4
Difference			+17.68		+2.39		+5.79		+56.6

long survival period. The decline in serum calcium occurs despite the persistently elevated concentration of plasma citrate. If one compares the 24- and 96-hour plasma concentrations of citrate and calcium one sees that despite similar plasma concentrations of citrate the serum calcium values are approximately 2.5 mg. lower 96 hours after nephrectomy. From these relations it is apparent that hypocalcemic factors develop in uremia that prevail over the hypercalcemic effect of citrate.

The marked increase in serum inorganic phosphorus probably causes the decline in serum calcium that occurs in uremia. It is interesting to note that there was a slight but significant decline in the serum inorganic phosphorus value associated with the injection of citrate into the nephrectomized dog. At the same time there was a delay in the otherwise continuous elevation of the serum non-protein nitrogen. These changes in serum inorganic phosphorus and non-protein nitrogen are probably not explained by hemodilution due to the injection of citrate and may reflect a temporary suppression of catabolic processes occurring in the tissues. The average survival time of the citrate-injected nephrectomized dogs was 120.5 hours.

As part of another study 3 other dogs were nephrectomized and treated similarly during the first 24 hours postoperatively as were the 9 animals already presented. The changes in the composition of the blood occurring in all 12 animals are summarized in table 3. In all instances some degree of serum calcium and citric acid

elevation occurred following nephrectomy. The extent of rise in the concentration of citrate and calcium were correlated with one another. The animals that showed the least rise in plasma citrate showed the least rise in serum calcium, and the animals that had the greatest rise in calcium showed the greatest rise in plasma citrate value after nephrectomy.

Effect of Bilateral Ureteral Ligation and of Citric Acid Injection into Ureteral Ligated Dogs. These experiments were necessary to distinguish between the metabolic and excretory functions of the kidney. The animals were injected for one hour with 80 mg/kg. of citrate/hour, 48 hours after ureteral ligation. Data on these animals, shown in table 4 indicate that serum calcium and citric acid were practically unchanged 24 and 48 hours after ureteral ligation, while the serum inorganic phosphorus and non-protein nitrogen had increased in a fashion comparable to that produced by nephrectomy. Injected citric acid caused a transient elevation in plasma citric acid concentration and an increase in the serum calcium value. However, the plasma

TABLE 4. EFFECT OF BILATERAL URETERAL LIGATION AND CITRIC ACID INJECTION UPON SERUM VALUES OF DOGS

NO. OF DOGS	POSTOPERATIVE HOURS	CITRIC ACID	CALCIUM	INORGANIC PHOSPHORUS	NON-PROTEIN NITROGEN
		mg. %	mg. %	mg. %	mg. %
6	Initial	3.41	10.56	4.52	32.0
6	24	4.60	10.57	10.89	90.3
6	48	4.36	10.14	15.22	156.2
5	49 ¹	58.87	12.23	15.26	160.1
5	72	6.06	9.14	23.66	247.6
2	96	8.82	8.66	29.15	272.5

¹ End of injection.

citrate and calcium values rapidly decreased to levels that approached the pre-injection concentration. The decrease in plasma citrate concentration 24 hours after injection was quite striking as compared to the decrease that occurred in a similar period of time in the nephrectomized dog that was injected with citric acid. The blood citrate injection was determined in 2 of the ureteral ligated animals 2 hours after the completion of citrate injection. The 2-hour plasma citrate decrease in one animal was from 46.4 mg. per cent to 12.5 mg. per cent and in the other animal from 20.85 to 10.50 mg. per cent. The 3-hour post-injection decrease in the nephrectomized animals was relatively slight despite the fact that they received only half as much citrate intravenously.

DISCUSSION

The foregoing experiments indicate that the transient hypercalcemia that occurs following nephrectomy is related to the plasma citric acid level. Furthermore, that elevation of the plasma citric acid level results from loss of an ability to remove citric acid that is present in the renal parenchyma. Participation of renal parenchyma in the removal of circulating citrates is clearly demonstrated by comparing the spontaneous

plasma citric acid values that occurred in the 2 groups of dogs as well as by their relative capacities to destroy injected citric acid.

The relatively slow rate of destruction of injected citrate in the nephrectomized dog is surprising in view of the presumed participation of this substance in the degradation of various organic acids throughout the body. Martensson's evidence (7) that the kidney is the site of concentration and excretion of citric acid has been referred to previously. It has also been proposed that the liver is the site of breakdown of citric acid (23). The difference in the findings and response of nephrectomized and ureteral ligated dogs leaves little doubt that the kidney is the principal site of removal of circulating citrates.

The plasma citrate level is determined by the relative rates of ingress and egress of citrates. It is not possible to decide at this time whether or not there is an increased ingress of citrates into the circulation following removal of the kidneys. Nor is there any evidence as to the site of origin of the citrate that is present in the plasma of normal or nephrectomized animals. There is considerable variability in the degree of plasma citrate elevation following nephrectomy. Some dogs may have a rise to as much as ten times the initial value 24 hours after nephrectomy, while others may have but a slight increase. This variability of response could be caused by a difference in the rate of destruction of citric acid by extra-renal tissues. The rate of removal of injected citrates varies considerably in different nephrectomized animals. It may be that the variability in plasma citrate rise following nephrectomy results from fluctuations in citrate release by some part of the body. Conceivably this source may be the bones acting in response to stimuli derived from the parathyroid gland or some other endocrine mechanism.

SUMMARY

Bilateral nephrectomy resulted in a definite increase in serum calcium and plasma citrates in 12 adult dogs 24 hours after the operation. Citrate injected intravenously into the nephrectomized dogs caused an elevation of the plasma citrate level which persisted for 2 or 3 days or until death. The serum calcium level was elevated in the nephrectomized dog but tended to decline as uremia progressed despite the persistence of an elevated plasma citrate level. Bilateral ureteral ligation in 6 adult dogs failed to cause a significant increase of either calcium or citric acid in the blood. Intravenously injected citrate was rapidly removed from the circulation of dogs with bilateral ligation of the ureters. It is concluded that the kidney is the principal site of removal of circulating citrates and that the transient hypercalcemia following nephrectomy is caused by an impaired ability on the part of the body to remove circulating citrates.

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EFFECT OF THYROPARATHYROIDECTOMY AND VITAMIN D UPON SERUM CALCIUM AND CITRIC ACID OF NORMAL AND NEPHRECTOMIZED DOGS

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IN THE preceding paper it was shown that a transient increase in serum calcium and citric acid occurs after bilateral nephrectomy but not after bilateral ureteral ligation. Since the principal body stores of both substances are contained in bone, it seems probable that the blood changes reflect mobilization from this reservoir. It is reasonable to suppose that the parathyroid glands are involved in the removal of calcium and citrate from bone following nephrectomy, particularly in view of Alwall's (1) findings that parathormone injection caused a rise in the blood citric acid as well as calcium.

The present study was undertaken to determine the effect of thyroparathyroidectomy upon the serum calcium and citric acid of normal and nephrectomized dogs. Observations have also been made on the effect of vitamin D administration on the serum calcium and citric acid of normal animals and those made hypocalcemic by thyroparathyroidectomy.

PROCEDURES AND RESULTS

In acute experiments the thyroid and parathyroid glands were removed at the time of nephrectomy. Four parathyroid glands were identified in the extirpated tissue of each animal included in this paper. In the chronic experiments the thyroparathyroidectomy was performed some weeks prior to nephrectomy. These animals were allowed to go untreated until tetany developed and then the serum calcium was restored to normal range by administration of vitamin D (10,000 IU/kg. daily), after which nephrectomy was performed as previously described. Similar amounts of vitamin D were administered to normal animals prior to nephrectomy to determine whether or not the hypercalcemic effect of the vitamin would influence the trend of events following nephrectomy.

The chemical methods and substances determined were as previously described (2). The diet used throughout this study consisted of bread, milk and a commercial dog food.

Effect of Nephrectomy and Parathyroidectomy upon the Serum Calcium, Inorganic Phosphorus, Non-protein Nitrogen, and Citric Acid Values of Fasting Dogs. The range of variation and average results obtained on 5 dogs are shown in table 1. These results show that the average increase in blood citric acid for the 5 animals was essentially the same as after simple nephrectomy. (See table 3 of preceding paper.) However, the elevation in calcium was less than after a simple nephrectomy,

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the average increase being near the limits of accuracy of the method for estimating serum calcium. Also, the increase in serum inorganic phosphorus was less than in the simple nephrectomized animal, whereas the rise in non-protein nitrogen was essentially the same in both groups.

The effect of citric acid injection on the serum values of 3 dogs 48 hours after operation is shown in table 2. It is apparent from the result that it is possible to raise the calcium value by citric acid injection in the thyroparathyroidectomized-nephrec-

TABLE 1. EFFECT OF THYROPARATHYROIDECTOMY AND NEPHRECTOMY AT ONE OPERATION ON SERUM VALUES OF DOGS

DOG NO.	CITRATE		CALCIUM		INORGANIC PHOSPHORUS		NON-PROTEIN NITROGEN	
	mg. %		mg. %		mg. %		mg. %	
	Initial	24-hour	Initial	24-hour	Initial	24-hour	Initial	24-hour
82	5.40	31.0	10.88	12.17	5.11	10.50	39.9	102.0
80	5.0	28.65	11.74	12.60	4.06	7.84	35.0	86.1
87	3.15	21.80	12.01	12.22	5.11	5.74	42.7	78.4
89	3.60	19.35	11.50	10.36	4.90	10.29	44.1	107.8
96	2.50	14.40	10.04	10.61	4.27	8.54	36.4	86.8
Average.....	3.84	21.93	11.03	11.59	4.58	8.58	39.4	92.3

TABLE 2. EFFECT OF CITRIC ACID AND CALCIUM INJECTION ON SERUM VALUES OF NEPHRECTOMIZED-PARATHYROIDECTOMIZED DOGS

POSTOPERATIVE HOURS	NO. OF DOGS	CITRATE	CALCIUM	INORGANIC PHOSPHORUS	NON-PROTEIN NITROGEN
		mg. %	mg. %	mg. %	mg. %
Initial	3	4.28	11.44	5.11	41.3
24	3	26.40	12.19	8.12	90.3
48	3	7.85	8.19	11.38	155.3
49 ¹	3	60.50	8.92	11.79	171.7
53 ²	3	60.70	13.23	14.53	176.4
72	3	3.70	9.35		

¹ End of citrate injection. ² Two hours after injection of 20 mg/kg. of calcium as calcium chloride.

tomized animals although the elevation is relatively less when the magnitude of increase in the blood citrate level is considered. The development of tetany in these animals after citrate injection necessitated the injection of calcium and prevented further observation on the calcium-raising effect of citric acid.

Effect of Vitamin D on the Response of Dogs to Thyroparathyroidectomy and Nephrectomy. By means of vitamin D administration hypercalcemia was produced in 5 animals prior to thyroparathyroidectomy and nephrectomy. These animals received 7000 I.U. of vitamin D/kg/dog until the serum reached a value of approximately 15 mg. per cent. The thyroid and parathyroid glands and kidneys were then removed and the blood constituents were followed. The average values on these

animals are shown in table 3. It will be noted that the hypercalcemia produced prior to operation was accompanied by a definite rise in the blood level of citric acid. A close association was noted between the serum calcium and citric acid values of the individual animals during development of the hypercalcemia. The average non-protein nitrogen and inorganic phosphorus were also somewhat elevated by vitamin D in hypercalcemic amounts. Following the operation citric acid rose but the calcium value fell during the first 24 hours by 2.19 mg. per cent. The preoperative vitamin D hypercalcemia resulted in higher serum calcium values 72 hours after operation as

TABLE 3. EFFECT OF VITAMIN D AND OF THYROPARATHYROIDECTOMY AND NEPHRECTOMY UPON SERUM VALUES OF DOGS

NO. OF DOGS	BEFORE AND AFTER OPERATION	CITRATE	CALCIUM	INORGANIC PHOSPHORUS	NON-PROTEIN NITROGEN
		mg. %	mg. %	mg. %	mg. %
5	Normal	4.15	10.68	4.37	35.1
5	Initial ¹	8.42	14.75	4.97	41.8
5	24 hr.	21.88	12.56	9.13	101.2
5	48 hr.	14.86	11.37	11.39	105
5	72 hr.	5.68	9.07	17.28	224.1

¹ Preoperative after vitamin D administration.

TABLE 4. BLOOD SERUM VALUES OF HYPERVITAMINOSIS D AND OF URETERAL LIGATED THYROPARATHYROIDECTOMIZED DOGS

NO. OF DOGS	BEFORE AND AFTER OPERATION	CITRATE	CALCIUM	INORGANIC PHOSPHORUS	NON-PROTEIN NITROGEN
		mg. %	mg. %	mg. %	mg. %
2	Normal	4.85	11.34	4.75	29.75
2	Initial ¹	5.35	13.70	5.10	43.50
2	24 hr.	3.30	10.76	9.07	100.75
2	48 hr.	2.70	8.35	10.33	175
1	72 hr.	2.15	8.44	17.00	223

¹ Preoperative after vitamin D administration.

compared to a group of animals reported previously (3) that did not receive vitamin D. The 72-hour serum calcium value for the previous group of animals was 5.9 mg. per cent compared to 9.02 mg. per cent for the present group of animals. However, the rate of descent was practically identical in the 2 groups: 5.7 mg/72 hours for the present group and 5.6 mg. for the previous group.

Table 4 shows results on two vitamin D hypercalcemic thyroparathyroidectomized dogs, the ureters of which were ligated at the same operation. The blood citric acid in these animals decreased during the first 24 hours following the operation. There was also a considerable decrease in their serum calcium values. The rate of decline of the serum calcium is similar to that of the nephrectomized animals despite the more gradual decrease in the serum citric acid of the latter group.

Effect of Chronic Thyroparathyroidpriva on the Responses to Nephrectomy. The

experiments thus far reported herein entailed loss of parathyroid and renal function within a few minutes of one another. There was a chance for an effect of secretion, already liberated from the parathyroid glands, to act during the subsequent survival period. To avoid this possibility thyroparathyroidectomy was carried out in an additional group of 8 animals. Tetany was allowed to develop in most instances and then vitamin D was given in sufficient amounts to restore the serum calcium values to normal. Usually 3 to 5 weeks had elapsed before the dosage of vitamin D was suitably adjusted to give approximately normal serum calcium values. At this time the kidneys were removed and the behavior of the serum constituents was followed. The results of this study are shown in tables 5 and 6.

TABLE 5. EFFECT OF THYROPARATHYROIDECTOMY AND VITAMIN D UPON SERUM VALUES OF DOGS

DOG NO.	DAYS POST-OPERATIVE	CALCIUM	CITRATE	INORGANIC PHOSPHORUS	DOG NO.	DAYS POST-OPERATIVE	CALCIUM	CITRATE	INORGANIC PHOSPHORUS
		mg. %	mg. %	mg. %			mg. %	mg. %	mg. %
48	0	11.44	3.55	4.83	132	0	9.51	3.20	4.69
	1	7.97	1.65	6.37		2	5.96	2.35	6.44
	2	6.43	2.25	6.37		11	12.25	4.35	5.67 ¹
	3	5.25	2.25	8.37	134	0	10.89	4.25	4.90
	5	5.12	1.50	7.56		5	4.68	2.80	5.88
	12	11.54	3.85	6.30 ¹		20	9.76	4.05	6.00 ¹
111	0	10.23	3.70	4.76	144	0	11.79	6.85	7.14
	3	7.76	1.90	5.04		1	7.21	3.70	6.65
	25	10.92	3.10	1		7	10.83	7.15	6.72 ¹
127	0	10.65	4.50	4.06		28	15.45	9.00	5.95 ¹
	5	5.18	2.25	5.81	172	Initial	10.96	6.75	3.78
	23	15.39	7.0	4.55 ¹		Pre-op.	11.73	5.85	5.39 ¹
	36	11.92	4.45	5.18 ¹		1	8.93	2.50	4.55 ¹
						8	15.03	10.00	5.25 ¹

¹ Vitamin D.

Table 5 illustrates the effects of thyroparathyroidectomy on the serum calcium and citric acid of adult dogs. Variations in serum calcium were usually associated with corresponding changes in citric acid. In every instance the citric acid content of the blood declined as the calcium content declined. When vitamin D was administered and the serum calcium rose the citric acid of the blood also increased. This was true in both hypocalcemic and hypercalcemic animals. In one instance (*dog 172*) vitamin D was given before parathyroidectomy to see whether or not the postoperative response would be altered.

In table 6 are shown the effects of nephrectomy on the serum values of these animals. About half of the animals had normal serum calcium values at the time of nephrectomy, whereas the other 4 animals had serum calcium values above the normal concentration. Six of the animals showed a decrease in serum calcium fol-

TABLE 6.
EFFECT OF NEPHRECTOMY UPON SERUM CALCIUM AND CITRATE VALUES OF VITAMIN D-TREATED THYROPARATHYROIDECTOMIZED DOGS

DOG NO.	BODY WGT.	DAYS AFTER PARA-THYROIDECTOMY	TOTAL VIT. D GIVEN	CITRATE mg. %				CALCIUM mg. %				INORGANIC PHOSPHORUS mg. %				NON-PROTEIN NITROGEN mg. %							
				Normal	Initial	24 hr.	48 hr.	72 hr.	Normal	Initial	24 hr.	48 hr.	72 hr.	Normal	Initial	24 hr.	48 hr.	72 hr.					
48	15	34	1,800,000	3.55	4	3.25	1.60	1.25	11.44	9.90	10.52	7.21	4.83	4.90	6.93	4.62	7.21	37.1	54.6	98	163.1	236.6	
15	15.3	34	2,300,000	3.70	2.50	3.85	1.50	1.35	10.23	9.88	10.09	8.49	4.76	4.62	9.38	11.69	16.10	40.6	36.4	70	114.1	185.5	
111	15.3	48	1,350,000	4.50	3.25	15.00	5.50	2.20	10.65	10.45	9.80	8.75	8.20	4.06	5.53	5.67	6.16	33.2	37.1	65.8	55.2	128.1	
127	15.4	48	1,275,000	3.20	3.55	10.35	5.80	6.75	9.51	10.80	9.76	8.08	7.40	4.69	5.74	4.09	6.44	37.8	81.2	131.6	175.9		
132	18.6	20	1,275,000	3.20	3.55	10.35	5.80	6.75	9.51	10.80	9.76	8.08	7.40	4.69	5.74	4.09	6.44	37.8	81.2	131.6	175.9		
134	17.7	26	2,500,000	4.25	4.25	6.40	1.40	1.70	10.89	12.96	11.05	8.40	7.04	4.90	5.39	7.84	8.89	16.31	46.2	42.7	74.9	106	
144	17.5	52	2,550,000	6.85	4	1.90	1.60	1.75	11.79	12.31	8.84	8.65	7.71	4.14	6.37	9.52	8.33	53.9	51.5	53.9	104.3	105.1	
172	18.5	24	800,000	6.75	6.75	5.15	4.60	2.25	10.96	13.41	11.24	9.02	8.03	3.78	6.09	8.12	8.05	11.34	37.1	70	113.4	145.6	
170	16	26	3,150,000	4.25	5.20	9.00	4.25	5	12.18	14.28	10.31	8.14	5.04	5.04	9.80	9.80	10.24	45.67	45.2	66.5	142.1	236.6	
Average.....				4.63	4.20	10.60	3.31	2.77	10.96	11.75	10.33	8.47	7.71	4.52	5.51	7.73	7.94	11.65	38.5	43.7	75.9	119.4	186.3

lowing nephrectomy. One animal had an increase of 0.62 mg. and one animal had values practically identical with the preoperative level. The plasma citrate values increased significantly in only 3 animals following nephrectomy and the remaining 5 animals showed little or no change from the preoperative level. The increase for these 3 animals was less than the average increase for simple nephrectomized animals contained in the previous paper. The serum calcium on these 3 animals decreased following the operation; therefore, there was no association between citric acid increase and serum calcium change in this group of animals.

The serum inorganic phosphorus of this group increased at a slower rate than after simple nephrectomy or concomitant thyroparathyroidectomy and nephrectomy. In some instances the 48-hour serum values were no greater or even less than the 24-hour values. The rise in non-protein nitrogen was also less in this group of animals.

DISCUSSION

If one compares the citric acid increase that occurs in animals subjected to concomitant thyroparathyroidectomy and nephrectomy (table 1) with that which follows simple nephrectomy (1), it is apparent that the order of change is the same and in this respect the 2 groups are indistinguishable. However, there is less serum calcium increase in the first-mentioned group, which suggests that at least a part of the post-nephrectomy hypercalcemia is dependent upon the function of the parathyroid glands in some sense other than that related to the

blood citric acid increase. The fact that the operation does not immediately free the body of the secretion of the extirpated endocrine glands tends to cast doubt on the interpretation of data obtained immediately after surgery. The difference in the response to nephrectomy of the acute and chronically parathyroprivic animals substantiates the impression that in the previous group parathyroid function was manifest after the glands had been removed. The principal differences in the response of the acute and chronically thyroparathyroprivic animals to nephrectomy were that the last-mentioned group manifested less tendency to an increase in serum citric acid and that it had no clear-cut instance of hypercalcemia following nephrectomy. The post-nephrectomy rise in serum citric acid observed in 3 dogs may have been caused by incomplete removal of parathyroid tissue in the chronically parathyroprivic animals.

When vitamin D was administered both to normal and parathyroprivic animals in sufficient amounts to raise the serum calcium there was a rise in serum citric acid as well. Hypercalcemia was usually accompanied by an elevation of citric acid in the blood to values above the normal range for that animal. Even though there was a constant relation between the mobilization of citric acid and calcium from bone, the blood concentration would still be subjected to variable influences such as destruction and excretion. In the nephrectomized animals these variables are reduced but not excluded.

The parallelism between changes in citric acid and calcium produced by vitamin D in both normal and parathyroidectomized dogs suggests that its mode of action must entail a mechanism affecting both substances. The simplest explanation of this action would be an increase in the liberation of calcium and citrate from their osseous reservoir. In a previous paper it was shown that an increase in serum calcium produced by calcium chloride injection did not cause the serum citrate to rise (2). From this finding it might be argued that the rise in serum calcium following vitamin D administration was produced by a mechanism involving citric acid rather than that the rise in citric acid was an effect of the increased serum calcium. However, it should be pointed out that duration of hypercalcemia following calcium chloride injection is brief as compared to that produced by vitamin D administration.

The decline in serum calcium and citric acid values following thyroparathyroidectomy may indicate in the case of citric acid as it does for calcium the lesser extent to which this substance is mobilized from the skeleton. Whether or not the activity of the kidney in citric acid metabolism is altered by thyroparathyroidectomy is not known. As in the normal animals, the thyroparathyroidectomized ones fail to show a rise in serum citric acid after ureteral ligation.

SUMMARY

The increase in serum calcium and citric acid that follows nephrectomy has been studied in relation to the functional activity of the thyroid and parathyroid glands. Concomitant thyroparathyroidectomy and nephrectomy resulted in an increase in blood citric acid similar to that produced by simple nephrectomy. Results obtained on 8 dogs thyroparathyroidectomized and maintained on vitamin D for a few weeks before nephrectomy yielded results which indicate that both the serum

calcium and citric acid increase following nephrectomy are dependent upon the thyroid-parathyroid apparatus. The parallel changes in serum calcium and citric acid produced by large doses of vitamin D suggest that its action influences the metabolism of both substances.

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ANTIPROTEOLYTIC ACTIVITY OF SERUM FROM DOGS WITH EXPERIMENTALLY-INDUCED PEPTIC ULCERS¹

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ANTIPROTEOLYTIC activity has been observed in the serum from animals of many species (1, 2). The degree of this reaction varies from species to species, but is fairly constant within a given species except in mice, where definite strain and sex variations have been observed (3), and in guinea pigs (4). Experimentally, a moderate elevation of the antiproteolytic titre of the serum of rabbits, dogs and mice has been produced by injecting or feeding trypsin (4, 5). Since the general response of an animal to intravenous trypsin is similar to histamine shock, it was suggested that the same antiproteolytic response might be produced by intravenous histamine injections. Dogs receiving large single doses and repeated smaller doses of aqueous histamine failed to show any increase in the antiproteolytic reaction of the serum (6). Prolonged administration of histamine in beeswax was found to produce an increased antiproteolytic activity in the serum of some dogs but not in others. The data reported herein indicate that the rise in antiproteolytic reaction is produced as a result of peptic ulcer formation rather than the histamine itself. This interesting response to ulcer formation may be of value in the study of peptic ulceration.

METHODS

Fifteen mongrel dogs, debarked and on a standard diet of Purina Dog Chow and water *ad libitum*, were given daily intramuscular injections of histamine⁴ in beeswax carrier prepared after the method described by Code and Varco (7). The total number of injections varied from 7 to 71. The antiproteolytic titre of the serum was determined, by a method previously described (8, 9), before beginning injections of histamine and then daily (except Sunday) in 6 dogs, and at intervals of 2 to 5 days in 9 dogs. The titre obtained with reconstituted pooled human plasma (PK) was used as a control, and the titres obtained with the serum of the dogs are reported in this series as percentage of the PK. The normal range, using tests on over 100 dogs at various times of day, has been found to be 100 to 120 per cent of PK. Upon death or sacrifice of the animals, postmortem examinations were carried out and microscopic sections of the stomach, duodenum and esophagus were obtained. Final diagnosis was based upon the microscopic sections.

RESULTS

The preliminary titres of all 15 animals fell within the normal range (100-120% of PK). Nine of the 15 dogs treated with histamine in beeswax showed a

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⁴ Histamine donated by Pfanstiehl Chemical Co. and Hoffmann-La Roche, Inc.

definite rise (140% of PK) in the antiproteolytic reaction of their serum. Two of these animals died on the day following and a third died 5 days after the initial rise in titre. At postmortem examination, these 3 dogs (*dogs 1, 3, 5*) were found to have acute perforated duodenal ulcers (figs. 1, 4). Two additional animals (*dogs 2, 4*)



Fig. 1. STOMACH AND DUODENUM of *dog 3*. Pathology: acute duodenal ulceration with perforation.

Fig. 2. STOMACH AND DUODENUM from *dog 13*. Pathology: chronic duodenitis.

Fig. 3. STOMACH from *dog 4*. Pathology: acute pre-pyloric ulcer and antral gastritis.

were killed on the day of the initial rise in titre when they were apparently perfectly well and were found at postmortem examination to have acute prepyloric and duodenal ulcers but without perforation or evidence of peritonitis (figs. 3, 5). Four animals (*dogs 6, 7, 8, 12*) had significant rises (140% of PK or greater) in titre with subsequent return to normal (100–120% of PK) during the course of injections.

These animals were killed after the return to a normal titre (in 2 animals after a single elevation, in one after 2 periods of elevation, and in another after 3 periods of elevation), and were found to have chronic organizing, or healed duodenal or esophageal ulcer and one had a chronic perforated esophageal ulcer which had been closed over by adjacent lung and pleura. An example of the titre of these animals can be seen in figure 6.

The 6 remaining animals (*dogs 9, 10, 11, 13, 14, 15*) failed to show a significant rise in antiproteolytic titre after 11 to 71 daily doses of histamine in beeswax. Three

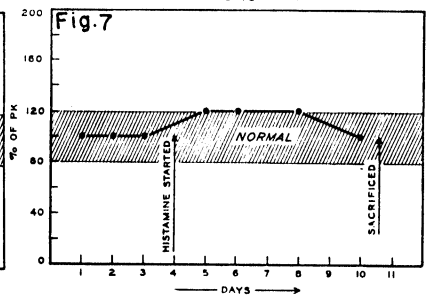
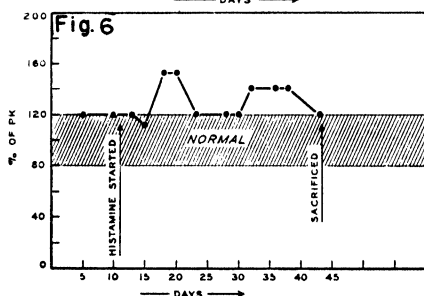
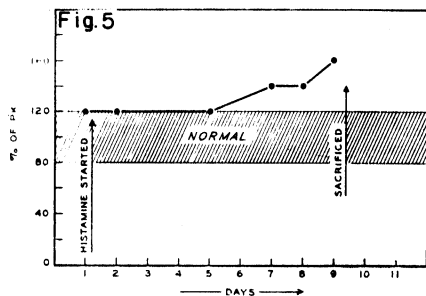
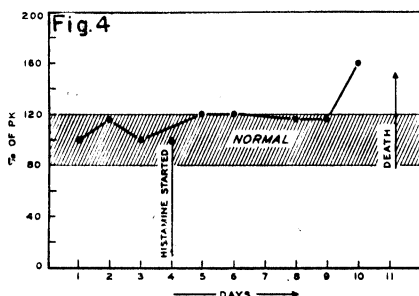


Fig. 4. ANTIPROTEOLYTIC TITRES of serum from *dog 3*. Pathology: acute duodenal ulceration with perforation.

Fig. 5. ANTIPROTEOLYTIC TITRES of serum from *dog 4*. Pathology: acute pre-pyloric ulcer and antral gastritis.

Fig. 6. ANTIPROTEOLYTIC TITRES of serum from *dog 11*. Pathology: organizing duodenal ulcer.

Fig. 7. ANTIPROTEOLYTIC TITRES of serum from *dog 13*. Pathology: chronic duodenitis.

of these animals had no lesions, 2 had chronic duodenitis without ulceration and one had chronic duodenal and esophageal ulcers 3 to 4 mm. in diameter, at postmortem examination (figs. 2, 7). The animal (*dog 14*) with the small ulcers did not have daily titres, and could have had an elevation in the 5 days intervening between tests.

All 6 animals with a titre of greater than 140 per cent of PK at the time of death had acute pathology (fig. 8). *Dog 6*, the titre of which was 140 per cent of PK at death exhibited both acute and chronic erosion of an area of the first duodenum. The 9 animals with a titre below 140 per cent of PK at death had chronic ulcer, duodenitis or were without demonstrable lesions. The rise in titre preceded clinical signs (anorexia, vomiting, gross melena and hematemesis) in 2 of the animals and

occurred at approximately the same time in the other four. It is interesting that one of the animals was given 71 daily injections of 30 mg. of histamine in beeswax yet failed to show a definite rise in titre. Barium studies of the stomach and duodenum of this dog on 2 occasions during the course of injections failed to show any evidence of ulceration, and at postmortem examination, no evidence of an ulcerating lesion could be found.

DISCUSSION

Data collected indicate that dogs with acute peptic ulceration produced by histamine in beeswax show an elevation of the serum antiproteolytic activity. Since acute peritonitis causes a rise in this activity (6), it might be presumed that the

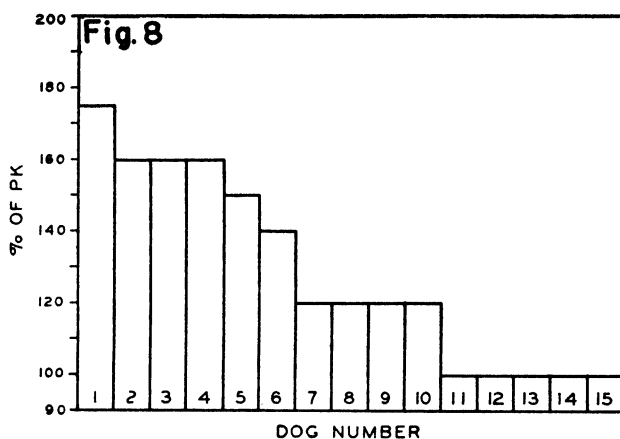


Fig. 8. LAST TITRE before death in % PK. Pathology: *Dog 1.* acute perforated duodenal ulcer; *dog 2.* acute duodenal ulcer; *dog 3.* acute perforated duodenal ulcer; *dog 4.* acute gastric ulcer; *dog 5.* acute perforated duodenal ulcer; *dog 6.* acute and chronic duodenal erosion; *dog 7.* chronic perforated esophageal ulcer; *dog 8.* organizing duodenal ulcer; *dog 9.* chronic duodenitis; *dog 10.* no pathology; *dog 11.* chronic duodenal and esophageal ulcers; *dog 12.* organizing duodenal ulcer; *dog 13.* chronic duodenitis; *dog 14.* no pathology; *dog 15.* no pathology.

elevation shown in these animals was due to such a lesion, and this presumption could not be denied on the basis of the first 3 animals which died after perforation of their ulcers. However, the 2 animals killed at the first significant elevation of titre, when they were to outward appearances perfectly well, were shown to have acute ulcers without any evidence of peritoneal reaction even in the immediately adjacent area. In addition, the dogs with chronic ulcers sacrificed a few days after elevations of titre showed no evidence of peritonitis.

From data collected on *dogs 6, 7, 8 and 12*, which showed one or more periods of elevated titre followed by a return to normal, it may be suspected that the chronic ulcers demonstrated followed acute ulcers formed at the time of elevation of titre. In these animals, chronic ulcers present at the time of death did not cause an increase in the antiproteolytic activity. Since every animal dying or killed with an elevated titre did have an acute ulcer, it seems justified to conclude that these animals had an

acute ulceration with each period of elevated titre. How the ulcer produces an elevated titre is unexplained. It is possible that the elevation is due to the release of protein breakdown products in the blood from digestion of the gastric or duodenal wall, since these materials are antiproteolytic *in vitro* (10, 5). However, it is difficult to conceive that this small amount of protein digestion could release substances in greater quantity than is the case during the digestion of a large protein meal, when the animals show no significant elevation of titre. Furthermore, ulceration of a much greater extent, such as in varicose or radiation ulcers, fails to elicit a similar response in the human patient unless associated with infection and fever (4). Mice injected with trypsin occasionally develop sterile abscesses and ulcers, without significant elevation of the titre above that due to the trypsin (4).

The sharp rise in the antiproteolytic reaction of the serum at the time of ulceration offers an objective method of determining the time of early ulceration, as shown by the 2 dogs killed on the first day of significant rise in titre. On the assumption that each elevation in antiproteolytic titre is associated with an acute ulceration, these data lend support to the theory that chronic ulcer is secondary to repeated acute ulceration.

SUMMARY

From the present study, it is apparent that histamine in beeswax, as such, has no definite effect on the antiproteolytic activity of the serum. However, when an acute gastroduodenal ulceration occurs as a result of this treatment, there is a sharp rise in the antiproteolytic titre. This elevation is relatively short-lived and, if the dog survives, the titre returns to the normal range. All of the dogs sacrificed when titres had returned to normal had chronic or organizing ulcers; whereas dogs that were sacrificed at the time of the elevation had acute ulcers. Those animals which showed no significant elevation of daily titres during the course of injection were found to have no ulceration at postmortem examination. A satisfactory explanation for the rise in titre in acute ulceration is lacking at present.

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EFFECT OF INTRAVENOUS SODIUM SULFATE ON RENAL EXCRETION IN THE DOG¹

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THIS report of 25 experiments on 5 dogs (15.6 to 23.5 kg.) concerns some urinary functions of the dog kidney which became evident during the course of steady intravenous infusions of solutions of sodium sulfate.

PROCEDURES AND METHODS

A wing tip, soft rubber catheter was introduced into the bladder of a perineotomized female dog. The animal was allowed to stand in a stall with minimal restraint and urine was collected at hour or half-hour intervals as previously described (1). Analyses were performed on 6 hourly urine specimens to determine pH , bicarbonate, chloride, sodium, potassium, magnesium, inorganic phosphate and inorganic sulfate. Serum was obtained during a control hour before starting intravenous infusion and during the 5th hour of infusion, and was analyzed for the same substances as the urine except that sulfate and pH were not determined. Infusions of solutions of sodium sulfate were carried out at either a 'fast' rate (ca. 4.19 cc/min.) or at a 'slow' rate (ca. 0.45 cc/min.) by means of a pump which forced the fluid through a plastic tube which lay in a superficial leg vein, and which was taped in place. Control infusion fluids, lacking sodium sulfate, were 5½ per cent glucose solutions. Other procedures and the methods of chemical analysis have been noted previously (1).

RESULTS

Figure 1 illustrates the essential equality of intake and output (isorrhea) of sodium and of sulfate which can be reached within 3 hours, and maintained. Fifth hour rates of excretion are actually slightly in excess of intake rates but presumably this overcompensation can only be temporary. All solutions tested, except those given at very low rates of intake of sodium sulfate, attained isorrhea for sodium and for sulfate. A minimal isorrheic quantity (MIQ) for this salt can be estimated (1) to be about 50 μ Eq/min. There is no evidence for the existence of a limiting isorrheic quantity (LIQ) in these data since no rate of intake was so rapid as to fail of being matched by excretion. Figure 2 provides evidence for the existence of both a minimal and a limiting isorrheic concentration (MIC, LIC) for sodium (ca. 50 and 420 mEq/l., respectively) and for sulfate (ca. 50 and 480 mEq/l., respectively). It also indicates the specific diuretic effect of sodium sulfate as distinguished from the water in the infusion since u/i values rise when the concentrations of sodium and sulfate in the

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infusion fluid are above their LIC. Figure 3 indicates that infusions of sodium sulfate have the effect of increasing the rate of excretion of potassium, calcium, and magnesium above control values, i.e. above those rates of excretion which occur with infusions lacking sodium sulfate. It is not determined that these effects are specifically due to the salt infused or to some more general osmotic stimulation (1).

DISCUSSION

Our results lead us to raise the following points, some of which have received little previous attention.

I. *Low Threshold Sodium*. Ordinarily sodium is the only cation which has a high threshold of retention and its content in the plasma is substantially electroneutralized by equivalent amounts of high threshold chloride and medium threshold bicarbonate. When exogenous sodium is administered in a salt such as sodium sulfate, that sodium exists completely electroneutralized by low threshold sulfate (2). The observations

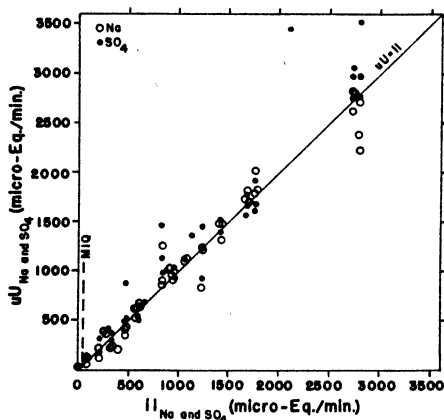


Fig. 1. RATE OF EXCRETION of sodium and of sulfate (uU_{Na} and uU_{SO_4}) plotted against intake rate of sodium sulfate (iI_{Na} and SO_4) at the 3rd, 4th, and 5th hours of the 'fast' series (rate of fluid intake, $i = 4.19$ cc/min.) and 4th and 5th hours of the 'slow' series ($i = 0.45$ cc/min.). The isorrheic diagonal passing through the origin is the locus of all points at which excretion rate equals intake rate ($uU = iI$). The points at $iI = 0$ are averaged of 16 determinations each. The minimal isorrheic quantity (MIQ) is indicated roughly to be 50 μ Eq/min. In general sulfate excretion is seen to exceed sodium excretion.

of Schwartz, Smith and Winkler (3) as well as those given here attest to the fact that the primary determinant of sodium excretion appears to be the concentration of sulfate in the urine when sodium sulfate is loaded on the body. A similar effect of low threshold potassium on high threshold chloride is seen when potassium chloride is loaded (4). A useful, if not completely general, rule can be stated to the effect that retention thresholds (of ions) are not uniquely characteristic and independent of associated ions. Sodium in the body consists of two fractions: high threshold sodium electroneutralized by high threshold anions; and low or no threshold sodium electroneutralized by low or no threshold anions. It is not necessary to postulate that sulfate, from administered sodium sulfate, lowers the threshold of endogenous sodium.

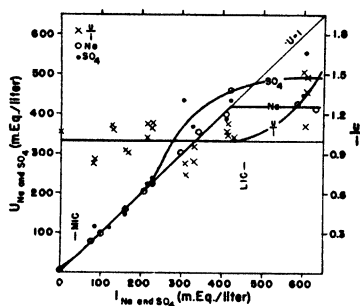
The observation made by Schwartz *et al.* (3) that sulfate excretion inhibits chloride excretion was not fully confirmed in the present experiments. The excretion rate of chloride was inhibited on some occasions but increased on others, particularly at high rates of sulfate infusion (table 1). However, the present results do not apply to simultaneously infused sulfate and chloride. Changes in chloride excretion may

merely reflect varying states of imbalance in chloride and/or other ions prior to and during experimental procedures.

When sodium sulfate was infused, the velocity constant, γ (= rate of excretion per minute per unit load of a substance), for sodium and sulfate averaged, respectively, 0.0212 and 0.0317 in the 'fast' series, and slightly higher in the 'slow' series. These contrast strikingly with γ_{Na} and γ_{Cl} which were less than 1/20 these magnitudes when sodium chloride was infused (4, 5). The contention is reaffirmed here that the excretion rate of a given ion from an infused compound depends in part, but importantly, on the electrically associated ion(s) of the compound (4). The force of this contention is to cast doubt upon the value of hypotheses concerning renal electrolyte excretion, edema formation, etc., which are framed simply in terms of a single, presumably 'dominant' ion (6-12).

II. *Osmotic Stimulation in Diuresis.* A concept of Verney (13-16) and others (17, 18) visualizes the basis of water diuresis to lie in the lowering of osmotic pressure

Fig. 2. RELATION BETWEEN URINARY CONCENTRATION of sodium and of sulfate (U_{Na} and SO_4) and concentration of sodium sulfate in infusion fluid (I_{Na} and SO_4) at 4th and 5th hours of 'fast' series (rate of fluid intake, $i = 4.19$ cc/min.). Also plotted for the same experiments on the same abscissa is the ratio of urinary flow to fluid intake rate (u/i). The diagonal passing through the origin is the locus of all points at which urinary concentration equals infused fluid concentration. Where the u/i curve rises above the horizontal line at $u/i = 1$, the ecretic effect of sodium sulfate is distinguished from that of water and the I value at that point is the approximate LIC. Where the sodium and sulfate curves leave or cross the diagonal $U = I$, isorrheic concentrations are again indicated, the minimal isorrheic concentration (MIC) at approximately 50 mEq/l. for both sodium and sulfate, the limiting isorrheic concentration (LIC) at approximately 420 mEq/l. for sodium, and the LIC at approximately 480 mEq/l. for sulfate.



of body fluids (when a positive load of water is established) which, acting as a stimulus to osmoreceptors in the central nervous system, results in a decreased output of the antidiuretic hormone of the posterior pituitary. The failure to attain appreciable diuresis following administration of isotonic sodium chloride may be supposed to follow naturally since no altered osmotic stimulation of osmoreceptors should be induced by this fluid. No attempt will be made here to review the many supporting and contradictory evidences which can be brought to bear on this elegant idea. We wish to note, however, evidence which is apparently at odds with this view, at least in its most simple form. From figure 2 we learn that the u/i values for all solutions of sodium sulfate below the LIC are substantially identical with those of water (5½ per cent glucose). That is, isotonic sodium sulfate, whose concentration is approximately 134 mM/kg. water (19), behaves not differently than plain water or isotonic glucose so far as diuresis is concerned. The difference in this behavior of isotonic sodium sulfate as compared with that of isotonic sodium chloride was early noted by Magnus (20). Since isotonic sodium sulfate is far below the LIC and the maximum urinary concentration (MUC), see section V, below), and since the same diuretic

effect occurs over a range of intake concentrations of from 0 to 480 mEq/l., there is little reason to believe that sodium sulfate in isotonic solution 'osmotically' resists tubular water reabsorption, including that which may be under the action of pituitary antidiuretic hormone.

No account will be given here purporting to explain the difference in diuretic behavior between isotonic sodium sulfate and isotonic sodium chloride. It may be doubted whether any substantial and rational explanation is actually to be found in current renal theory. However, it is of descriptive and possibly fundamental importance to observe that whereas in the case of sodium chloride solutions we deal with a solute having a high threshold of retention, we deal in the case of sodium sulfate solutions with a solute having an extremely low threshold. The complex and physiologically necessary interdependence of excretion between water and sodium chloride has been exhaustively examined previously, and quantitative relations have been stated in steady state equations (4, 21, 22). In these terms one can describe and predict the different diuretic activities of sodium chloride solutions of different given

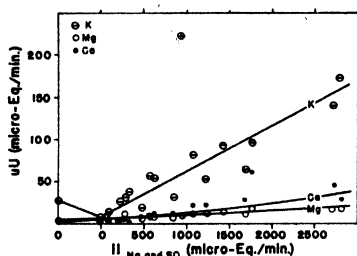


Fig. 3. RATE OF EXCRETION (μ U) of potassium, magnesium, and calcium at the 5th hour plotted against rate of intake of sodium sulfate (I_{Na} and SO_4). Points on the abscissal 0 at the extreme left, however, represent averages of all control excretion rates before the beginning of infusion (potassium, 25 cases; magnesium, 25 cases; and calcium, 22 cases). Points on the other abscissal 0 represent averaged excretion rates at the 5th hour of infusions containing no sodium sulfate but 5% per cent glucose (potassium, 8 cases; magnesium, 8 cases; calcium 7 cases). The increase in excretion rate at the 5th hour with the highest I_{Na,SO_4} values is of the order of 25-fold for calcium, 20-fold for magnesium,

and 10-fold for potassium, when compared with the 5th hour excretion rate during glucose infusion ($I = 0$). 'Slow' and 'fast' series combined.

concentrations. Isotonic sodium chloride is merely one of an infinite number of salines of such differing diuretic potency. With sodium sulfate, by virtue of its having no appreciable threshold, there is no necessary interdependence of the excretions of water and solute so long as urinary concentrations remain below the LIC. Thus we could well expect that isotonic sodium sulfate solution would act no differently in regard to diuresis than an equal volume of water alone. Just as no threshold phenol red (23) or low threshold calcium (1) excretions are independent of water excretion, so is that of low threshold sodium sulfate. The fact that γ_{Na} and γ_{SO_4} are essentially the same in both 'slow' and 'fast' series further attests to the independence of solute and solvent excretion.

We have observed that injection of pitressin during the diuresis of isotonic sodium sulfate reduces urine flow but it is planned that these and related studies be reported later. At this point, however, it is concluded that 'isotonic' solutions, by virtue of their tonicity alone, have no necessary critical significance for renal excretion without reference to the particular solute involved.

III. *Tubular Maximum Hypothesis of 'Osmotic' Diuresis.* Wesson, Anslow, and Smith (11, 12) have proposed an osmotic mechanism to account for mannitol diuresis.

In this view, it is assumed that sodium (and chloride) reabsorption in the proximal tubule is an active process accompanied by a passive back-diffusion of water along the osmotic gradient ($A_{Na} - U_{Na}$), thus produced² between luminal fluid and plasma. The presence of quantities of mannitol in the glomerular filtrate is thought

TABLE I. 'SLOW'¹ AND 'FAST'² SERIES. CONTROL³ AND 5TH HOUR DATA

I_{804}	i_{804}	TIME	uU_{PO_4}	uU_{Cl}	uU_{HCO_3}	L_{H_2O}	I_{804}	i_{804}	TIME	uU_{PO_4}	uU_{Cl}	uU_{HCO_3}	L_{H_2O}	u/i
'Slow' Series							'Fast' Series							
o	o	o	0.64	16.9	18.8		o	o	o	22.8	61.5	36.2		0.09
		5	3.80	6.30	1.43	-69			5	0.16	8.0	7.10	140	1.00
o	o	o	2.60	9.73	5.84		o	o	o	9.23	12.4			0.03
		5	5.13	5.98		3			5	4.26	4.26	15.0	257	0.992
o	o	o	14.3	37.8	1.05		o	o	o	15.7	154.	0.12		0.14
		5	5.20	6.90	0.76	-134			5	1.09	25.6	6.29	309	0.82
o	o	o	0.935	4.22	0.436		o	o	o	4.93	35.2	16.1		0.13
		5	0.22	0.60		27			5	0.30	43.4	37.3	-80	1.01
244	90.4	o	0.395	9.34	20.5		82	336	o	1.63	10.7			0.04
		5	3.43	15.5	13.8	-73			5	0.99	13.2	0.17	267	0.805
564	205	o	0.26	10.6			120	486	o	1.14	30.9	6.46		0.07
		5	0.125	9.00	0.016	-89			5	10.1	17.5	1.75	28	1.08
1040	283	o	2.61	6.05			160	584	o	10.92	10.1			0.04
		5	3.64	0.467	8.15	-221			5	1.19	o	0.283	308	0.932
1672	612	o	3.02	15.9	30.2		211	844	o	5.58	4.40	0.456		0.04
		5	10.1	45.2	17.7	-351			5	36.0	o	5.58	261	1.12
2556	912	o	12.2	17.7	7.14		228	940	o	8.40	77.0	5.10		0.10
		5				-579			5	2.0	50	91.0	41	1.11
2980	1062	o	4.03	14.2			308	1240	o		7.85			0.04
		5	10.4	49.9	58.0	-576			5		12.1		423	0.826
							329	1425	o	7.8	33.8	3.9		0.04
									5	9.1	53.5	90.7	307	0.95
							411	1690	o	4.54	30.6	0.79		0.082
									5	4.34	69.5	39.1	-113	1.06
							420	1760	o	15.7	87.0	3.59		0.08
									5	5.70	52.6	145	95	1.04
							600	2742	o	0.638	5.28	7.45		0.03
									5	0.826	82.6	100.8	-481	1.51
							605	2800	o	1.66	67.9	9.25		0.07
									5	28.7	109.	135.	-191	1.38

¹ Rate of fluid intake, $i = 0.45$ cc/min.

² Rate of fluid intake, $i = 4.19$ cc/min.

³ o hr.

I_{804} = concentration of sodium sulfate in infused fluid (mEq/l.). i_{804} = rate of infusion of sodium sulfate (μ Eq/min.). uU_{PO_4} , uU_{Cl} , uU_{HCO_3} = respective rates of excretion of phosphate, chloride, and bicarbonate (μ Eq/min.). L_{H_2O} = net load of water (cc.) at the end of the 5th hour, i.e., total water infused - (total water excreted in urine + 120 cc. of estimated insensible water loss). u/i = ratio of rate of urinary flow (u) to rate of infusion of fluid (i).

to prevent the usual back-diffusion of water in the proximal tubule to such an extent that the unreabsorbed fluid, containing mannitol and some unreabsorbed sodium, floods the distal tubular transport system concerned with reabsorption of water and sodium. The result is increased excretion of these latter. These workers report that

² Where A_{Na} is plasma concentration and U_{Na} is urine concentration of sodium, in mEq/l.

even during high mannitol diuresis, the ($A_{Na} - U_{Na}$) gradient never went below 60 to 90 mEq/l. The proximal reabsorption of sodium was stated to be retarded by the development of this critical concentration gradient which occurs when the presence of mannitol in tubular urine prevents reabsorption of water and favors dilution of sodium in the urine. Some potential reabsorption of sodium is thus further hindered, and 'osmotic' diuresis is then attributed to the combined osmotic action of mannitol and unreabsorbed sodium.

The same osmotic system apparently does not serve for sodium sulfate. Where a specific diuretic (ecuretic) effect of this salt is in evidence above that of water, as in the 'slow' series (table 2) and in the 'fast' series where I_{SO_4} is greater than the LIC of sulfate (fig. 2), it is seen that at least as much or more sulfate than sodium is excreted (fig. 1). If we suppose sulfate to be a relatively unreabsorbed ion (2, 3, 24), it follows that in sodium sulfate diuresis there remains in the urine an electroneutralized block, mostly of sodium sulfate, which remains unreabsorbed. No sodium, un-

TABLE 2. 'SLOW' EXPERIMENTS¹ ILLUSTRATING URINARY FLOWS² AND URINARY CONCENTRATIONS³ OF SODIUM⁴ AND OF SULFATE⁵

I_{SO_4}	II_{SO_4}	U_{Na}	U_{SO_4}	u	I_{SO_4}	II_{SO_4}	U_{Na}	U_{SO_4}	u
0	0	5.0	11.0	0.700	564	205	530	720	0.250
0	0	56.0	40.0	0.166	1040	283	440	432	0.934
0	0	15.0	16.0	0.367	1672	612	470	488	1.38
0	0	12.0	300.	0.100	2556	912	490	496	2.24
244	90.4	520.	472.	0.250	2980	1062	540	556	2.08

¹ Rate of fluid intake, $i = 0.45$ cc/min. ² u (cc/min.) ³ mEq/l. ⁴ U_{Na} ⁵ U_{SO_4} .

I_{SO_4} represents the concentration of sodium sulfate in infused fluid. II_{SO_4} represents rate of intake of sodium sulfate (μ Eq/min.). The leveling off of urinary concentration and the increase of urinary flow, as the intake of sodium sulfate increases is clearly shown. Where $I_{SO_4} > 0$, average $U_{Na} = 498$ mEq/l. and average $U_{SO_4} = 527$ mEq/l., values equal to or greater than the LIC.

neutralized (or which could not be neutralized) by sulfate, remains. There is, therefore, a ($A_{Na} - U_{Na}$) gradient for 'free' sodium of 140 mEq/l., or more (when the plasma sodium is raised by sodium sulfate infusion). That is, no critical gradient, beyond which sodium is no longer actively reabsorbable, exists here. Mudge *et al.* (25) find this to be the case also in urea diuresis. Since the excretion of other cations than sodium is enhanced in sulfate diuresis (fig. 3, table 1), the dangers inherent in oversimplified 'dominant' ion hypotheses in renal physiology are again noted.

IV. *Stability of LIC of Sodium and Sulfate.* It has been observed that in certain instances where 'osmotic' diuresis occurs, larger urine flows tend to be accompanied by a falling MUC (26-28). In the present experiments (fig. 2, table 2) where loads and infusion concentrations of sodium sulfate did not press far beyond the physiological limit of the LIC, no evidence of falling U was found as I increased. The steady state equation (4, 21, 22) $\frac{I - A_r}{U - A_r} = \frac{u}{i} = \frac{I}{U}$ described the isorrhea and no need was evident under these conditions to apply differential equations to relate urine flow

to urine concentration (28)³. We conclude, without prejudice as to its mechanism of action, that sodium sulfate can be considered an isorrheic diuretic (4).

V. *Miscellaneous and Negative Results*. It has been ascertained in these studies (table 2) that the LIC for sodium is less than 498 and probably approximately 420 mEq/l. (fig. 2). That for sulfate is less than 527 mEq/l. (table 2) and is probably approximately 480 mEq/l. (fig. 2). The MUC for these, observed during one experiment when an infusion of hypertonic sodium sulfate was stopped before the 5th hour and urine flow was falling, was 530 for Na and 800 mEq/l. for sulfate.

Following infusion of hypertonic sodium sulfate there were regularly observed increases in serum sodium concentration and decreases in serum chloride, potassium, phosphate, calcium and magnesium. The effects on the four latter ions, however, were not appreciably different from those found when no sodium sulfate was contained in the intake fluid. No regular influence of infusion was observed on either serum bicarbonate or urinary pH. Rüdél (29) reported an alkalinizing and Eggleston (30) an acidifying influence on the urine during sodium sulfate diuresis. In our experiments (table 1) urinary bicarbonate showed some tendency to increase with higher rates of sodium sulfate infusion as compared with lower or zero rates of sodium sulfate infusion.

SUMMARY AND CONCLUSIONS

During steady intravenous infusions of solutions of sodium sulfate in dogs, it was found that equality of intake and output (isorrhea) of sodium and of sulfate can be reached within 3 hours, and maintained. A minimal isorrheic quantity (MIQ) for these ions is ca. 50 μ Eq/min. No limiting isorrheic quantity (LIQ) was found. A minimal isorrheic concentration (MIC) for sodium and for sulfate was estimated at 50 mEq/l.; a limiting isorrheic concentration (LIC) for sodium was estimated at 420 mEq/l.; and an LIC for sulfate was estimated at 480 mEq/l. Maximum urinary concentrations (MUC) for sodium and for sulfate were found at 530 and 800 mEq/l., respectively.

The velocity constant of excretion for sulfate exceeds that for sodium when sodium sulfate is infused, e.g., $\gamma_{Na} = 0.0212$; $\gamma_{SO_4} = 0.0317$. These values are more than 20 times greater than $\gamma_{Na \text{ or } Cl}$ when sodium chloride is infused. It is suggested that high threshold ions in the body may have a low threshold fraction, e.g. that fraction of sodium which is or could be electroneutralized by sulfate. The fact that isotonic sulfate behaves diuretically like plain water, as contrasted with isotonic sodium chloride, is discussed in the light of the posterior pituitary hypothesis of diuresis. Evidence is presented that hypotheses which purport to account for certain phenomena in renal physiology by reference to 'dominant' ions such as sodium, are too simple to account for known facts. A steady state equation can be used to describe the isorrhea of sodium sulfate under the conditions of these experiments. The effect

³ I , U are concentrations (mEq/l.) of sodium or sulfate of infusion fluid and urine, respectively; A_T is the threshold of retention (mEq/l.) whose value is almost zero for sodium and sulfate under these conditions; u/i is the ratio of rate of urine flow, u (cc/min.) to rate of infusion of fluid, i (cc/min.).

of infusions of sodium sulfate on renal excretion of several ions, and on their serum levels, is reported.

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ANOXIA IN EXPLOSIVE DECOMPRESSION INJURY¹

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EARLIER studies in this laboratory (1, 2) indicate that anoxic anoxia and aeroembolism represent major factors in the etiology of explosive decompression injury, and that the rapid expansion of intrapulmonary gases is of less importance in this type of trauma than has been generally supposed.

In an attempt at further evaluation of the influence of anoxic anoxia in the genesis of explosive decompression injury, over 250 rats were decompressed from normal, ambient atmospheric pressure to 21 mm. Hg (altitude equivalent 80,000 ft.) within an average time of 0.64 seconds. Following explosive decompression the animals were maintained under the reduced pressure for varying time intervals to ascertain the effects of different post-decompression periods at simulated altitude; recompression was then accomplished at the same rate as that of decompression. In other experiments rats were placed in an atmosphere of nitrogen at normal ambient pressures, while further tests were designed to determine the effects of variations in metabolic rate on survival. In the latter cases thyroxin (1 mg/day) was administered for 10 days prior to the tests, or rats were exposed to cold until an average deep rectal temperature of 31.6° was attained before they were subjected to decompression. In other experiments the effects of CO₂ and adrenaline were observed, while in a further group of animals the influence of thoracic constriction (taping) on survival was studied.

RESULTS

Time at Simulated Altitude. Table 1 shows the marked effect of varying the post-decompression time under reduced pressure. In an initial series of 20 rats, recompressed as rapidly as possible (> 1 second) following the decompression with consequent virtual elimination of the anoxic period, all survived without injury. When, however, animals were maintained under the reduced pressure for as short a period as 10 seconds, not all survived. The mortality rate was seen to increase in subsequent series, moreover, with increasing time at altitude, until a period of 40 seconds was reached, when all 12 rats succumbed. Figure 1 indicates the relationship between time at reduced pressure and mortality rate.

Both gross and microscopic examination of the lungs of the animals which succumbed showed the usual picture of pulmonary hemorrhage with alveolar rupture, such injury being particularly notable at the periphery of the tissues. The extent and number of such lesions, moreover, gave some evidence of correlation with the duration of the exposure to reduced pressure.

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The exposure of rats to an atmosphere of nitrogen at 760 mm. Hg for varying time periods yielded results in keeping with the above. Cessation of respiratory activity was used as the criterion of death, as in the earlier series. Thus, the mortality was found to be 40 per cent after 30 seconds of nitrogen breathing, and reached 100 per cent after a 50-second exposure to the gas. Mechanical factors involved in the two types of experiment indicated that the difference between lethal time under reduced pressure and that in nitrogen (10 seconds) might be considered as insignificant. Nineteen of the 22 rats employed in the nitrogen experiments (86%) exhibited pulmonary lesions at autopsy, and the results secured were interpreted as indicating the efficacy of anoxic anoxia in the production of injuries indistinguishable from those resulting from explosive decompression. In figure 1 may be noted the marked parallelism between simple anoxia and post-decompression stay at 'altitude' as regards the mortality rate.

Metabolic Effects. Since it appeared that anoxia represents a major factor in the etiology of explosive decompression injury, it was thought that alterations in oxygen utilization or availability should cause demonstrable changes in the mortality rate of animals maintained at altitude (anoxic environment) for different time periods

TABLE 1. EFFECT OF TIME UNDER REDUCED PRESSURE (21 MM. HG, ALTITUDE EQUIVALENT 80,000 FT.) FOLLOWING EXPLOSIVE DECOMPRESSION ON SURVIVAL

TIME UNDER REDUCED PRESSURE	NO. OF ANIMALS TESTED	MORTALITY	TIME UNDER REDUCED PRESSURE	NO. OF ANIMALS TESTED	MORTALITY
sec.		%	sec.		%
> 1	20	0	20	10	30
10	10	10	30	10	70
15	10	20	40	12	100

following explosive decompression. However, in experiments in which 10 rats were pre-oxygenated under a pressure of 2 atmospheres of the gas for 30 minutes, the mortality rate following subsequent decompression (and maintenance at altitude for 40 seconds) was not decreased, and all of these animals succumbed as in the experiments involving no pre-oxygenation. Since the amount of excess oxygen forced into the blood under these conditions (either in solution or in combination with hemoglobin) would be small, these experiments were not looked on as conclusively negative in character.

A further series of 58 rats was placed in a cold box for periods up to 30 minutes prior to decompression, resulting in an average drop in rectal temperature of 6°. This procedure was carried out under the assumption that such conditions would appreciably lower metabolic rate, and hence oxygen consumption. Reference to table 2 shows that these rats were indeed able to withstand an increased post-decompression stay at altitude when compared to the initial series. In fact the cooled rats were found to tolerate a time-at-altitude of 80 seconds without fatality, whereas all of the normal series succumbed after 40 seconds under reduced pressure and the first death occurred after a stay at altitude of only 10 seconds. The degree of 'protection' afforded by the cooling therefore approximated 100 per cent.

In order to effect an increase in metabolic rate, thyroxin was injected (1 mg/day) into 8 rats for a period of 10 days. The animals were then decompressed to 21 mm. Hg and maintained at reduced pressure for 20 seconds. All succumbed with typical pulmonary lesions. In a second series of rats similarly treated but kept in the colony for several days prior to decompression to allow the effects of thyroxin to abate, all survived. These results were interpreted as indicating that metabolic rate, and thus oxygen consumption, may influence the outcome of explosive decompression to a demonstrable degree, and hence they give added evidence of the importance of anoxia in the etiology of explosive decompression injury.

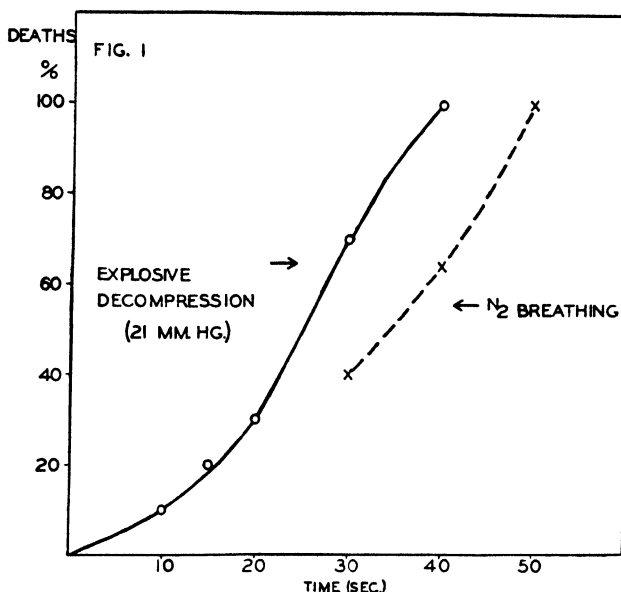


Fig. 1. EFFECT OF MAINTAINING RATS AT 'ALTITUDE' for different time intervals subsequent to explosive decompression. Note parallelism between resulting mortality rates in rats so treated and those produced by simple anoxia (nitrogen breathing).

With the thought that respiratory failure following decompression might depend to some degree on medullary anoxia, and since it has been shown by Kline (3) that carbon dioxide administration is effective in increasing altitude tolerance, a group of 10 rats were ventilated for 10 seconds with CO₂ and immediately (1-3 seconds) decompressed. Five of these animals (50%) survived this treatment, with maintenance at 21 mm. Hg for 40 seconds or the normally lethal time-interval. A further series of 10 rats was injected with adrenaline hydrochloride (0.06 cc., 1:1000) 60 seconds before being decompressed, and 4 of these animals survived a 40-second stay under reduced pressure. These findings were interpreted as indicating the efficacy of respiratory and cardiovascular stimulation as survival factors.

Thoracic Compression. In an earlier study (1) it had been found that tight thoracic taping gave some protection to rats explosively decompressed to a simulated altitude

of 50,000 feet. Whitehorn *et al.* (4), working with dogs, found no protective action attributable to such taping in similar experiments. A series of 10 rats, with the thorax taped to permit only minimal respiratory activity, was immediately decompressed to 21 mm. Hg. Eight of the animals survived the decompression and subsequent exposure to the lowered barometric pressure for 40 seconds, the normally lethal time-at-altitude. These findings confirmed the earlier tests on rats cited above.

DISCUSSION

Determination of the fundamental etiology of explosive decompression injury is of primary importance, since any device designed to minimize this hazard among aircrews must necessarily depend on a knowledge of the mechanics and cause of this type of insult. Earlier studies (2) had demonstrated the inadequacy of intrapulmonary gas expansion per se as the principal lethal agent, and these as well as other observations (5, 6) have indicated anoxic anoxia as a factor of major importance. In the present experiments the time-at-altitude studies were interpreted as

TABLE 2. EFFECT OF LOWERED BODY TEMPERATURE ON SURVIVAL FOLLOWING EXPLOSIVE DECOMPRESSION TO A PRESSURE OF 21 MM. HG. ANIMALS COOLED TO AN AVERAGE RECTAL TEMPERATURE OF 31.6° C

TIME UNDER REDUCED PRESSURE	NO. OF ANIMALS	MORTALITY	TIME UNDER REDUCED PRESSURE	NO. OF ANIMALS	MORTALITY
sec.		%	sec.		%
30	4	0	90	7	43
40	9	0	100	5	60
50	7	0	110	3	33
60	2	0	120	15	80
70	2	0	130	4	75
80	4	0	140	2	50

substantiating the relative ineffectiveness of gas expansion, in comparison with anoxia, as a major factor in the genesis of fatal injury.

The failure of pre-oxygenation to increase tolerance to post-decompression time at altitude is perhaps not surprising when one considers that the increased amount of oxygen which may be placed in solution by this method is very small (less than 5 volumes per cent) and that the hemoglobin can carry but little more of the gas in any event than under normal conditions. For these reasons the negative nature of these experiments was not considered as greatly significant.

The close agreement between the time of lethal exposure to nitrogen and to reduced barometric air pressure following explosive decompression further suggests that anoxia may well represent the primary lethal agent in such experiments. Thus, cessation of respiration was found to occur in 100 per cent of cases after 40 seconds at altitude following decompression, and after 50 seconds of nitrogen breathing at normal ambient pressures.

The experiments in which metabolism might be assumed to be altered (lowered body temperature, thyroxin administration) might be similarly interpreted, as well as those in which stimulation of the respiratory center and increased cerebral blood

flow (7, 8) were effected through inhalation of CO₂, or general circulatory stimulation secured by means of adrenaline. Although respiratory and cardiac failure may be brought about through nervous and other endogenous mechanisms not as yet investigated, it appears that anoxic anoxia must be considered to be a factor of major importance in the etiology of explosive decompression injury.

The beneficial effects obtained by means of externally applied thoracic pressure (taping) are not so readily explained. It is true that the thoracic wall of the rat is considerably more flexible than that of the dog, permitting a greater degree of constriction, which should produce greater limitation of pulmonary expansion through mechanical constriction in the smaller animal. However, the thoracic cage of man, like that of the dog, is comparatively rigid and inelastic, and it may be doubted that the thoracic bandage would offer an efficient or practical principle for aircrew protection.

SUMMARY

In experiments in which rats were explosively decompressed to 21 mm. Hg (altitude equivalent 80,000 ft.), subsequent time at this reduced pressure was found to be directly correlated with survival. Pre-oxygenation (2 atmospheres, 30 min.) did not materially affect survival following decompression. Alterations in metabolic rate (and inferentially, oxygen consumption) through exposure to cold or injection of thyroxin gave evidence that post-decompression survival was correlated with oxygen demand. Both CO₂ pre-breathing (respiratory excitation, cerebral hyperemia) and adrenaline injection (cardiovascular stimulation) were found to increase post-decompression tolerance of rats to the rarefied environment. These findings were interpreted as indicative of the major importance of anoxic anoxia as a causative factor in death resulting from explosive decompression injury.

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ELECTRICAL ALTERNATION IN EXPERIMENTAL CORONARY ARTERY OCCLUSION¹

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IN THE course of studies on the electrocardiographic changes found in intra- and extraventricular derivations in experimental coronary artery occlusion of the dog we have frequently observed electrical alternation. Changes in the T wave and ST segment have been described by previous authors (1-6). The high incidence of electrical alternation following coronary occlusion has not been previously stressed however.

METHODS

Eleven dogs weighing between 11 and 13 kg. were used in this study. They were anesthetized with sodium pentobarbital (25 mg/kg.). Under artificial insufflation of the lungs, the chest was opened, and through a 2-cm. incision through the anterior parietal pericardium the proximal portion of the left anterior descending coronary artery was carefully dissected. A non-constricting ligature was passed around the artery. As previously described (6, 7) an electrode was introduced into the left ventricle by way of the carotid artery (usually the right). The pericardial sac was fixed by sutures to the anterior chest wall, and the precordial leads were obtained by needle electrodes placed in the subcutaneous tissue overlying the left ventricle. In 3 dogs direct epicardial wick electrodes were used. Control records consisted of standard limb and augmented unipolar limb leads, and left intraventricular and epicardial or precordial leads, with a Wilson central terminal as the indifferent electrode. Continuous records were made simultaneously of intracavitary and epicardial leads.

The left anterior descending artery was occluded completely, without changing the position of the heart, for periods varying from 30 seconds to 32 minutes. Since electrocardiographic recovery was rapid after the release of the ligature, multiple experiments could be performed on the same animal, and a total of 33 experiments was performed on 11 dogs. (In every instance complete recovery, as evidenced by the stabilization of the electrocardiogram, occurred before another experiment was attempted.) An average interval of 23 minutes elapsed between experiments.

RESULTS

In addition to the characteristic T and ST-T changes (1-6) electrical alternation was detected in 8 of the 11 dogs in whom occlusion of the anterior descending branch

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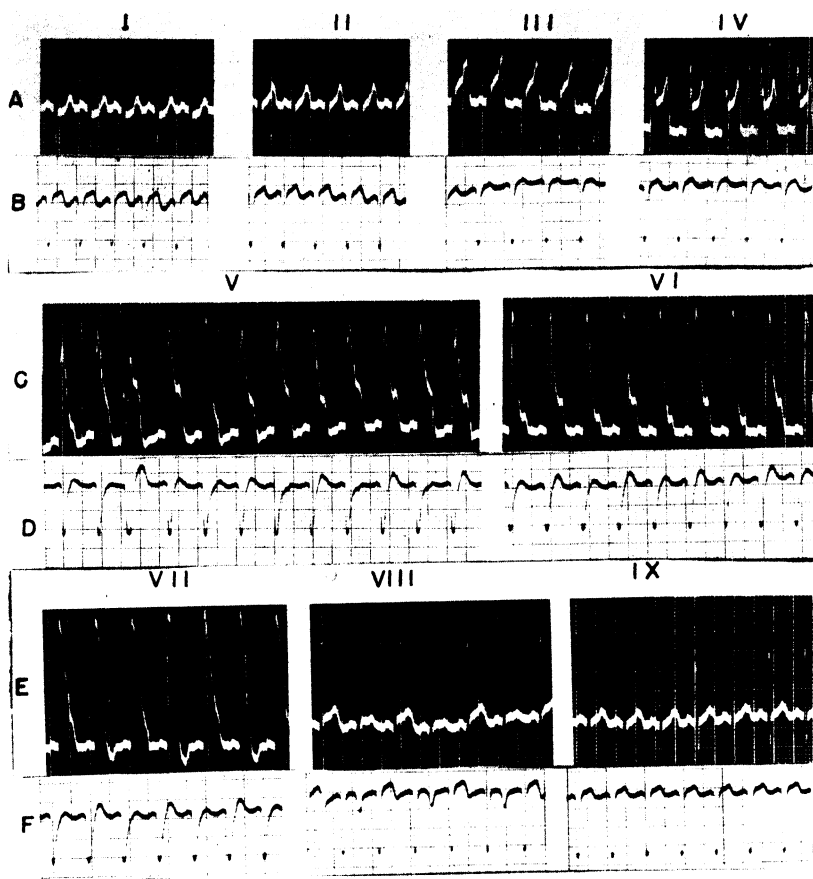


Fig. 1. ELECTRICAL ALTERNATION following occlusion of left anterior descending coronary artery. Rows A, C and E: epicardial surface of anterior left ventricle. Rows B, D and F: cavity of left ventricle. *Column 1*: control records; artery then occluded. *Column 2*: 25 seconds after occlusion; T wave in epicardial lead is taller. *Column 3*: 55 seconds. ST elevation in epicardial lead; cavity lead shows slight depression of ST junction and smaller T wave. *Column 4*: 105 seconds; marked ST elevation in epicardial lead. No change in cavity lead. *Column 5*: 132 seconds; electrical alternation, more marked after premature beat. Epicardial R wave shows increased amplitude, alternation and delay of intrinsic deflection. ST-T alternation (both leads) is discordant (see text); occlusion released at 138 seconds. *Column 6*: 243 seconds after release of occlusion. ST-T alternation persists; now concordant (see text). *Column 7*: 253 seconds; T of epicardial lead inverted in alternate beats. *Column 8*: 293 seconds; concordant ST and T alternation. Note alternately inverted T in cavity lead. *Column 9*: 7 minutes after release; complete recovery.

of the left coronary artery was performed. Of the remaining 3 dogs, one developed ventricular fibrillation 80 seconds after the occlusion; one dog had adequate functioning collateral circulation and showed only minimal signs of ischemia after coronary occlusion (slight ST elevation in the intracavitary tracing) which subsided

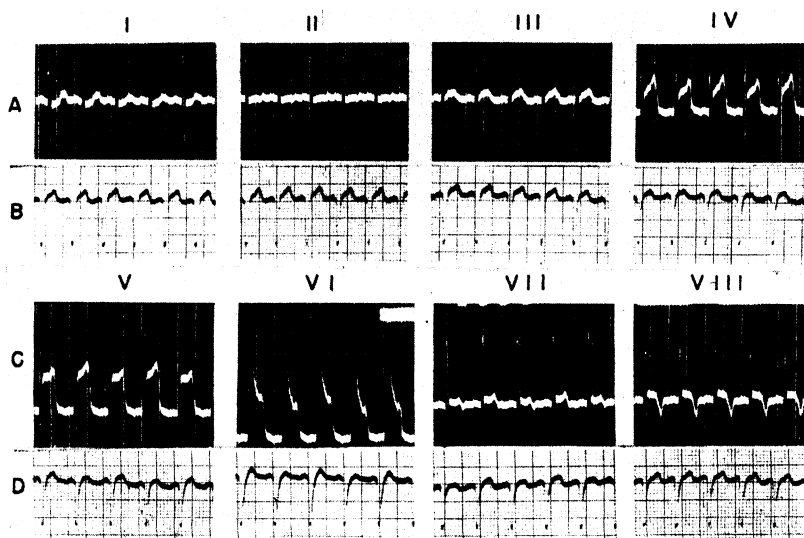


Fig. 2. ELECTRICAL ALTERNATION following occlusion of left anterior descending coronary artery. Rows *A* and *C*: wick electrode lead from anterior surface of left ventricle. Rows *B* and *D*: cavity of left ventricle. *Column 1*: control records; artery then occluded. *Column 2*: 10 seconds after occlusion; T in epicardial lead smaller and diphasic. *Column 3*: 20 seconds; epicardial T again upright. *Column 4*: 90 seconds; epicardial lead shows ST-elevation with slight, but definite alternation. Cavity lead shows slight alternation in contour of T; alternation is concordant. *Column 5*: 120 seconds; alternation more marked; now discordant; epicardial R taller. Continuous records show that apparent increase in elevation of ST is actually the result of T-R depression. *Column 6*: 190 seconds; alternation persists; QRS now wide; occlusion released. *Column 7*: 5 minutes after release of occlusion; less marked but definite, concordant alternation of ST and T. *Column 8*: 10 minutes after release; alternation has ceased. Residual ST elevation and T inversion in epicardial lead.



Fig. 3. ELECTRICAL ALTERNATION following occlusion of left anterior descending coronary artery; left intraventricular lead; continuous record. *Part A*: control. *Part B*: 10 minutes after occlusion of artery. Latter shows TQ depression and ST elevation from the isoelectric line. Following a premature beat (arrow) marked alternation of the ST-T complex begins. There is no QS change. Small *P* wave precedes each ventricular complex. Alternation persisted for 34 seconds following which runs of left ventricular premature beats occurred, terminating in ventricular fibrillation.

spontaneously although complete occlusion was maintained for 32 minutes; the third dog showed characteristic T wave changes and ST segment displacement after occlusion, with rapid recovery from each of 6 separate occlusions, lasting from 30 seconds to 7 minutes. Thus, of 9 dogs which survived coronary occlusion and de-

veloped electrocardiographic signs of coronary insufficiency, 8 (89 %) developed electrical alternation.

In establishing the diagnosis of electrical alternation we excluded the possibility of bigeminal rhythm due to ventricular premature systoles by noting the presence of a regular sinus rhythm and a *P* wave preceding each ventricular complex. The possibility of the effect of rapid respiratory rate was also excluded. When the respirations have a rate exactly one-half that of the heart beat a pseudo-alternation may

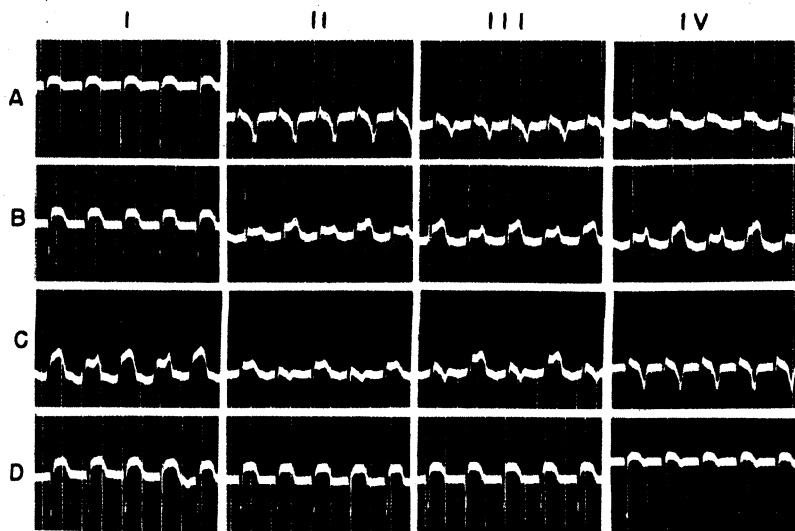


Fig. 4. ELECTRICAL ALTERNATION following occlusion of left anterior descending coronary artery. Leads from left ventricle: cavity and anterior epicardial surface. Row A—1st record: control from cavity. 2nd record: control from epicardium; inverted T caused by cooling of epicardium; artery then occluded. 3rd record: 30 seconds after occlusion; T less inverted. 4th record: 34 seconds; T now upright; slight but definite ST-T alternation. Row B—1st record: cavity lead 34 seconds after occlusion; ST is elevated; QS decreased. A few seconds later marked alternation of the ST-T complex appeared. 2nd and 3rd records: alternation of ST-T complex in epicardial lead; occlusion then released. 4th record: 12 seconds after release. Row C (epicardial lead) and row D (cavity lead): 4 records, left to right, made 100 seconds, 160 seconds, 14 minutes and 10 minutes after release of occlusion. Epicardial lead after initial further ST elevation (1st record) shows gradual decrease in ST elevation, then alternate T inversion, and finally return to control. Cavity lead, which in this experiment never recorded alternation, shows gradual decrease in ST elevation and, finally, a small negative T. Maximum rate change was from control of 154 beats per minute to 166.

be observed (8). This possibility was excluded by the artificial insufflation of our open chested preparation at a rate of 15 to 20 per minute, which quieted the respiratory muscles.

Changes in the T wave preceded S-T deviations in experiments in 7 dogs. In all but two of these experiments, the T wave in the epicardial leads became taller and more positive. This change was then followed by S-T segment elevation (fig. 1). In the remaining two experiments, T-wave inversion preceded the appearance of tall upright T waves (fig. 2). In 6 experiments on 5 dogs, however, S-T segment deviations

preceded T-wave changes although in other experiments on the same animals the opposite obtained. In all but two experiments the T waves in the intracavitary leads either became negative if originally positive, or more negative if originally negative. In two experiments the T wave became more positive. Although S-T segment displacement was always positive in the epicardial tracings, in intracavitary leads the direction of S-T segment deviation was not constant. In 3 animals the S-T segment was depressed, indicating essentially laminar subepicardial injury (6). In 6 animals the S-T segment was elevated due to injury extending to the subendocardium (fig. 3).

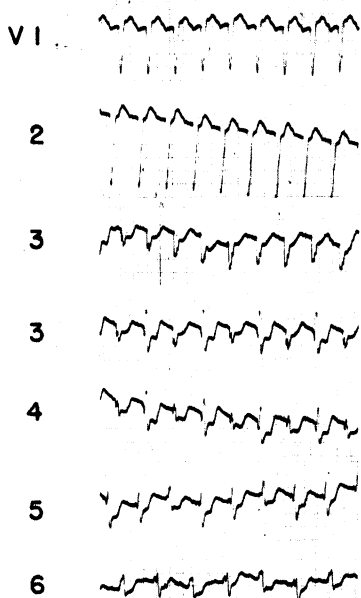


Fig. 5. ELECTRICAL ALTERNATION occurring during operation. Patient died 15 minutes later. Alternation appeared in V_3-6 ; alternately absent R wave in V_3 , diminutive in V_4 , and with a different form in V_5 and V_6 .

Electrical alternation appeared about 2 minutes after the coronary artery was occluded in all but two experiments; in one experiment electrical alternation occurred after the release of an occlusion of 2 minutes' duration; in another experiment after 10 minutes of occlusion. However, alternation did not appear in all dogs after the first occlusion. In 3 dogs, electrical alternation occurred from 84 to 120 seconds (an average of 99 seconds) after the first occlusion. In 3 dogs, alternation occurred on an average of 130 seconds after the second occlusion performed 20 minutes after the first experiment; and in 2 dogs, alternation occurred after the third occlusion. Thus, repeated coronary occlusions enhanced the development of electrical alternation. Green (9) similarly observed that frequent repetition of excessive prolongation of clamping of a main coronary vessel caused an annoying ventricular alternation, as manifested by aortic pressure curves. In our experiments electrical alternation was

variable in its duration, lasting from several seconds to 20 minutes with the artery occluded. In every instance, however, electrical alternation disappeared from 3 seconds to 5 minutes after the constricting ligature around the coronary artery was released.

Alternation of the ST-T and QRS complexes, alone or together, was observed. The most common was alternation of the ST segment, occurring in experiments on 7 of the 8 dogs which showed alternation. In the epicardial leads there was alternation in the extent of ST segment elevation. In intracavitary leads, in some instances there was alternation of positively displaced ST segments, and in others of depressed ST segments.

In several experiments alternation occurred in the epicardial but not in the intracavitary leads (fig. 4). Since the electrodes were not precisely juxtaposed, the exploring intracavitary electrode in the above instances was spatially oriented so that it was not influenced by the area producing alternation in the epicardial lead. The importance of the spatial relation of the exploring electrode to the theoretical surface at the junction of the injured and uninjured areas has been stressed previously (6).

Alternation was considered to be concordant when complexes with the larger ST-T areas were recorded in both epicardial and intracavitary leads simultaneously. In discordant alternation, the larger ST-T areas of the former coincided with the smaller areas of the latter, and vice versa (figs. 1, 2). In the course of an experiment on the same preparation the records were sometimes concordant at one time and discordant at another (fig. 1).

Alternation of the area of the T wave occurred both as to magnitude and direction. Alternation of displaced ST segments and of T waves occurred with and without concomitant QRS alternation. Alternation of the QRS complex involving amplitude and, rarely, the duration of QRS, when present, was slight. Thus while the areas of the QRS complexes varied less than 10 per cent, the ST-T areas varied on an average of 40 per cent between beats. In successive experiments in the same animal various combinations of the above types of alternations were often observed. Alternation of the Q-T interval was not observed.

Premature Beats. In 4 of 8 dogs alternation was immediately preceded by the appearance of a ventricular premature beat. In one dog alternation ceased completely after the appearance of a ventricular premature beat.

The heart rate was not significantly increased, so that electrical alternation in our experiments cannot be ascribed to the influence of rate (10).

DISCUSSION

Several excellent reviews on alternation of the heart have appeared in recent years (11-14). Although alternation has been described as occurring in intoxications, paroxysmal tachycardias, after cauterization of the myocardium, and after experimental coronary artery occlusion (5, 6, 10, 13, 15) it is not generally stated that alternation occurs as frequently as found in our series. Furthermore, the dominance of alternation of the ST-T complex without comparable QRS changes has not hitherto been emphasized.

Fundamentally, the factor underlying all forms of alternation is a marked

prolongation of the refractory phase of some part of the heart leading to alternating localized block (8, 11). Following a previous activation an impulse finds some regions of the myocardium still refractory. Consequently the response in every alternate beat will be abnormal—mechanically and/or electrically. Clinically, and experimentally, the alternation produced by the failure of excitation of one portion of the heart cannot be distinguished from alternation involving the entire myocardium of the left ventricle (9). Clinically, electrical alternation usually occurs where there are no other electrical signs of injury, and the areas of normal QRST vary considerably between beats (8).

In Wiggers' laboratory, Orias (16) demonstrated that after coronary artery ligation there occurred a primary depression of contraction and a decrease in ventricular systole. This was caused by a deletion of fractionate contractions in the ischemic (potentially infarcted) areas (17). The masses of cardiac fibers are not immediately deleted in toto, however, but rather in an alternate fashion. Employing the myocardiographic technique of Tennant and Wiggers, Green (9) has demonstrated that mechanical alternation occurs after experimental coronary artery occlusion, with the area of ischemia bulging alternately and producing an alternation of aortic pressures. Alternate diminution or absence of contraction in the ischemic area is responsible for the alternating pulse shown in the aortic pressure curve and myocardiographic tracing. Therefore, in our experiments, it is reasonable to assume that the electrical alternation of injury effects may similarly be caused by the failure of certain *fractions* of ischemic myocardium to respond on alternate beats. Thus, there may be a prolonged refractory period and a 2:1 partial heart block at the site of ischemia in the left ventricle. Alternate impulses penetrate the barrier, while others pass around it. Injury can be manifested by QRS, ST and T changes (6) and alternation may occur in any of these complexes at the time they show evidence of such injury.

Alternation in our experiments was predominantly of the ST-T complex, probably because injury currents of rest and activity persist longer than the transient changes in repolarization (T wave) and depolarization (QRS) (6). The magnitude of injury effects may vary considerably between beats, if *fractions* of the ischemic area fail to respond. Occasionally, however, alternation occurs in which injury effects are present in one beat and absent in the following one. It is possible that the ischemic area manifests injury effects of activity only, i.e., the involved area may not be depolarized by injury, but may become unresponsive during activation, giving rise to an injury current of activity (6). If the entire area of ischemia is alternately not excited, then injury effects would be absent in one beat and present in the following one. If alternation occurs in an area of old infarction alternation of the QRS complex and not of the ST-T results (fig. 5).

If the fraction of ischemic myocardium which fails to respond has an essentially laminar orientation, alternate tardiness of repolarization of the lamina would reverse or intensify the usual epicardial-endocardial gradient, depending on the location of the laminae. The subepicardial lamina normally recovers before the subendocardial lamina, explaining positive T waves in epicardial leads, and negative T waves in cavity leads (7). The epicardial-endocardial gradient would be reversed by tardy

repolarization of injured epicardial lamina and intensified by tardy repolarization of injured endocardial lamina. In the former instance, alternate tardiness of repolarization of the epicardial lamina results in alternation of the direction of T waves (fig. 1). In the latter instance, alternate tardiness of repolarization of endocardial lamina results in alternation of the magnitude of the T wave.

Alternation did not occur in all experiments after the first coronary occlusion but did appear on the second or third temporary occlusion. From this it is apparent that previous occlusions, although of short duration, predisposed to the production of alternation. The mechanism remains obscure. Although complete electrical recovery occurred between occlusions, structural changes or metabolic disturbances may well have persisted.

In our experiments the appearance of alternation cannot be attributed to the effects of acceleration of heart beat (15), since there was not a significant, consistent increase of rate. Alternation occurring while the heart is beating at normal rates indicates that the myocardium is badly damaged or dying.³

Previous investigators have similarly noted the onset of alternation following premature beats (12, 15, 18, 19), and have proffered different interpretations. Wenckebach (20) raised the question whether a dynamic form of alternation exists in which changes of ventricular filling rather than abnormalities of the myocardium are solely concerned. This view received some support from Wiggers in the case of temporary alternation following a long diastolic pause. All the phenomena associated with alternation were accounted for by changes in diastolic size and arterial resistance (21). In studies of the contraction of ischemic regions, however, Green, employing myocardiographic technique, showed that the time required for recuperation rather than dynamic changes in diastolic size or tension was the factor dominantly concerned in evoking such alternation. Thus, following a premature beat, there are inherent changes in the muscle which lead to contractions in alternate beats only. Changes in initial tension and length are therefore not to be regarded as fundamental determinants of alternations (9). Since in our experiments cessation of alternation following a premature beat was also noted, and since alternation occurs without a preceding premature beat, the exact significance of the premature beat in the genesis of alternation under the conditions of our experiments is indeterminate. However, we do not deny that in hearts so predisposed, a premature beat will often reveal alternation or exaggerate it (15).

SUMMARY

Electrical alternation developed in 8 of 9 dogs (89%) which survived coronary artery occlusion and developed electrocardiographic signs of myocardial ischemia. Electrical alternation occurred within 2 to 3 minutes after occlusion and was transient. Repeated temporary occlusions of a coronary artery predisposed to the de-

³ After the completion of this report, an interesting case demonstrating this point was observed. During a local operative procedure, R. Z., a 55-year-old man with old myocardial infarcts, complained of severe angina pectoris, and then developed electrical alternation (fig. 5). The patient died 15 minutes later. Autopsy revealed infarction of anterior, apical and interventricular portions of left ventricle with multiple organized thromboses of both coronary arteries. Death was due to acute superimposed upon chronic coronary insufficiency.

velopment of electrical alternation. Under similar circumstances, mechanical alternation has also been observed by Green. Alternation was predominantly of the ST-T complex, although alternation of the QRS complex, and of the T wave (in direction and amplitude) was also noted. It is postulated that in our experiments, electrical alternation of injury effects is due to the failure of certain fractions of ischemic myocardium to respond on alternate beats.

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TEMPORAL SUMMATION OF STIMULI STUDIED WITH THE AID OF ANTICHOLINESTERASES^{1, 2}

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DALE and his associates recognized the rigid conditions which integration of muscular activity imposes on the theory of cholinergic stimulation of muscle. They wisely postulated a need of rapid destruction of acetylcholine liberated at motor end plates (1). Opponents of the theory of neurohumoral mediation, however, believed that acetylcholine could not be destroyed with sufficient speed at synaptic endings to permit the nervous system to function as a transmitter of nerve impulses. Nachmansohn, on the other hand, revealed an effective cholinergic enzyme system sufficiently rapid, in his opinion, to permit the nervous system to function as a transmitter of nerve impulses (2). The fact, however, that anticholinesterases potentiate rather than weaken central nervous activity has put the combined issue of synaptic transmission of nerve impulses and humoral mediation in a paradoxical situation; for if the central nervous system functions as a transmitter of nerve impulses, and if transmission of nerve impulses depends upon speedy destruction of acetylcholine, how is potentiation of nervous activity to be explained by retardation of destruction of acetylcholine?

This paradoxical confusion, as we see it, stems from a general acceptance by humors and electro neurophysiologists of the theory of synaptic transmission of nerve impulses, the validity of which is unsubstantiated by experimental evidence. Substitution of the theory of neurocellular generation of nerve impulses for that of synaptic transmission of nerve impulses would seem to clarify the issue (3, 4). Accumulation of acetylcholine in bombarded neurons would, in accordance with the theory of neurocellular generation of nerve impulses, strengthen neurocellular generating currents and consequently potentiate central nervous activity. Retardation of destruction of acetylcholine is not only compatible with potentiation, but may constitute an important mechanism of nervous integration.

The carotid nerve offers attractive opportunities for studying the nature of potentiation and of temporal summation of stimuli. As is well known, this nerve plays a significant role in the normal regulation of breathing. When artificially or normally stimulated it produces highly coordinated hyperpnea in which inspiratory and expiratory contractions are appropriately strengthened. Dual excitation is thought to be caused by a division of chemoreceptor afferents into 2 branches, an inspiratory branch terminating on inspiratory neurons and an expiratory branch terminating on

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expiratory neurons as illustrated in figure 1. Such termination of sensory afferents would explain the simultaneousness of temporal summation of repetitive stimulation of the carotid nerve seen in figures 2, 3, 4 and 5. Since these records show a predominating reinforcement of inspiratory contractions, 4 synaptic terminations have

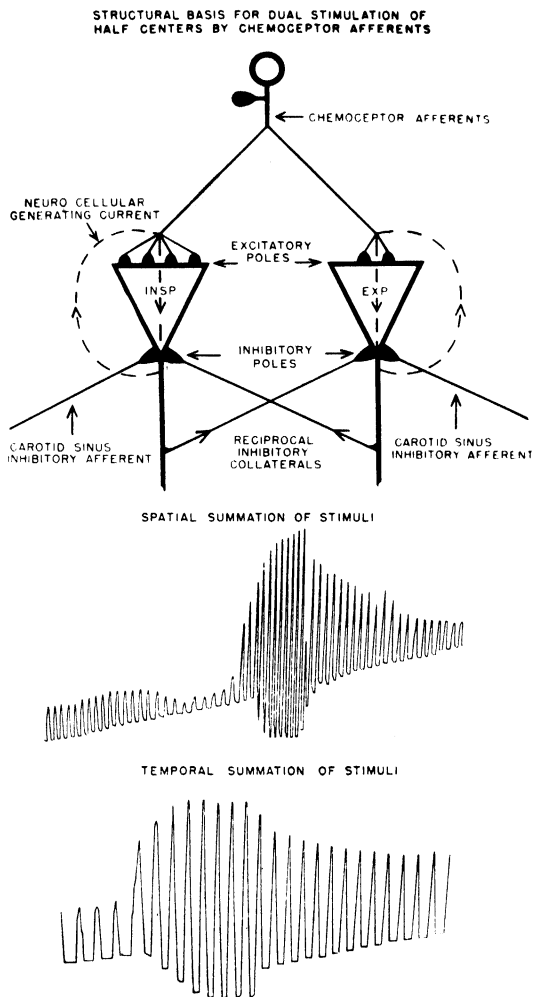
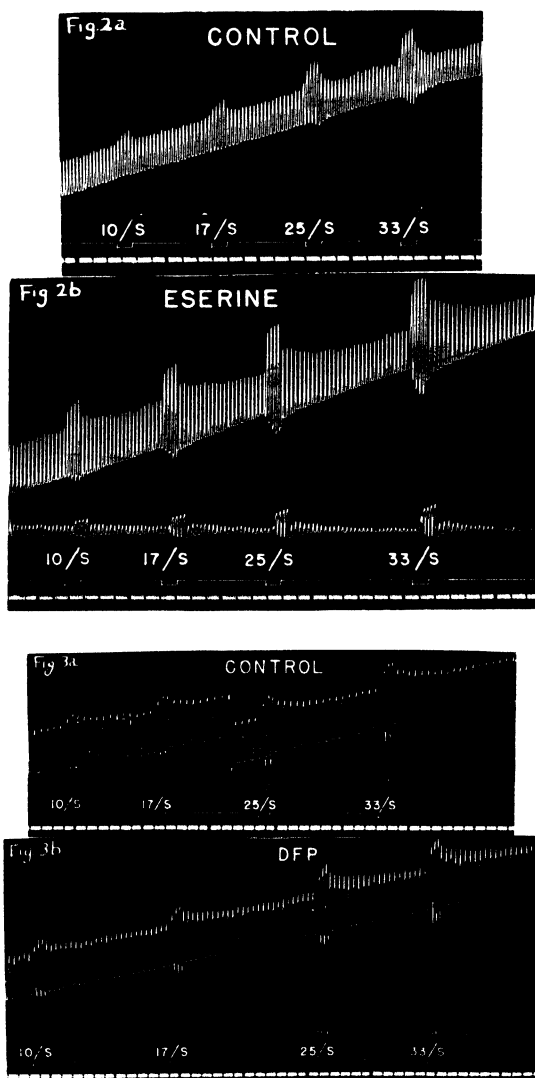


FIG. 1. *Upper Schema* shows a hypothetical structural arrangement consistent with dual excitation of inspiratory and expiratory half-centers, predominant stimulation of the inspiratory half-center and simultaneousness of temporal summation of stimuli at opposing half-centers when the carotid nerve is stimulated. *Middle Record* is a respiratory response to faradic stimulation of the carotid nerve in which strength of stimulation is progressively increased from left to right. Spatial summation of inhibition is evident in the left half of the tracing where stimulation of the inhibitory afferents from the carotid sinus predominates. Spatial summation of excitation replaces that of inhibition in the right half of the tracing where stimulation of the excitatory afferents from the chemoreceptors gains ascendancy. *Lower Tracing* shows temporal summation of stimuli kept at uniform intensity. The similarity of respiratory response during spatial and temporal summation suggest the basic similarity of these 2 phenomena. For details see text.

been arbitrarily indicated for each inspiratory branch and only 2 for each expiratory branch (5).

Temporal summation of stimuli, in our opinion, is very similar to spatial summation of stimuli and like spatial summation can be interpreted in terms of the neurohumoral theory. The final increase of respiratory response to simple increase of

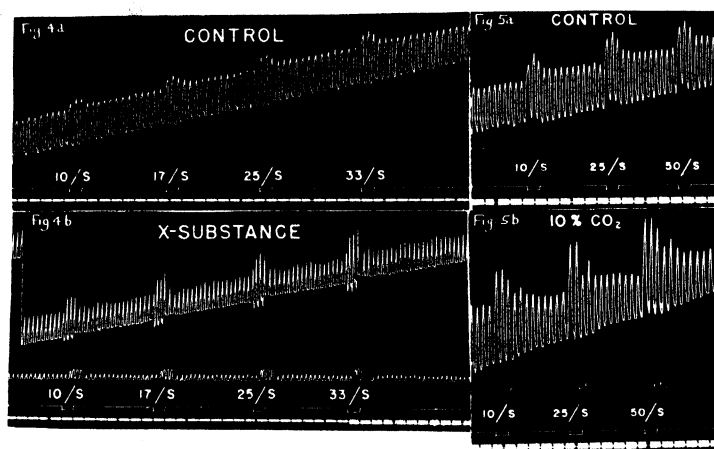


FIGS. 2 AND 3. TEMPORAL SUMMATION of stimuli of several frequencies before and after administration of anticholinesterase. Torsal respiratory movements are recorded with the aid of rebreathing tank and Hutchinson spirometer. Facial respiratory movements are recorded immediately below spirometer tracing by simple suspension. Time is recorded in 1 and 10-second intervals.

strength of stimulation of the carotid nerve, such as illustrated in figure 1, is assumed to be caused by stimulation of an increased number of chemoceptor afferents, by a greater liberation of neurohumor and by a resultant intensification of neurocellular currents which generate nerve impulses. In other words, spatial summation is an

expression of an increase of electrochemically active surface resulting from recruitment of activity of additional synaptic endings.³

Temporal summation of stimuli, in contrast, could theoretically occur without the aid of increase of electrochemically active surface and could be an expression solely of an increase of intensity of existing electrochemical activity. This increase of intensity of electrochemical activity could be a resultant of several factors, all related to time: 1) speed of enzymatic destruction of acetylcholine, 2) duration of periods of destruction of acetylcholine intervening between stimuli, and 3) duration of period of stimulation or continuance of accumulation of undestroyed acetylcholine at nerve centers. It is along these lines that we attempt to analyze the nature of temporal summation in the description of the experiments which follows.



FIGS. 4 AND 5. TEMPORAL SUMMATION of stimuli of several frequencies before and after administration of anticholinesterase. Torsal respiratory movements are recorded with the aid of rebreathing tank and Hutchinson spirometer. Facial respiratory movements are recorded immediately below spirometer tracing by simple suspension. Time is recorded in 1 and 10-second intervals.

METHODS AND RESULTS

The carotid nerve was stimulated at frequencies of 10, 17, 25 and 33 stimuli/second with a square wave generator. A current-strength of slightly super threshold value was used in all experiments. The nerve was dissected with care and protected against drying to maintain a uniform excitability to stimulations over extended periods of time. A short period of stimulation of 10 seconds was selected to permit rapid recovery of reflexes between stimulations.

A typical set of responses of breathing to repetitive stimulation of the carotid nerve at 10, 17, 25 and 33 stimuli/second is illustrated in the control records of figure 2. It will be seen that temporal summation of stimuli occurred at all frequencies of

³The initial decrease of respiratory response to simple increase of strength of stimulation of the carotid nerve is assumed to be due to stimulation of increasing numbers of inhibitory sinus afferents ending at the inhibitory poles of the respiratory neurons. (See legend of fig. 1.)

stimulation and that the magnitude of summation increased with each increase of frequency.

It may be tentatively suggested that increase of frequency of repetitive stimulation causes greater accumulation of acetylcholine in the respiratory neurons. This could come about in 2 ways: by a simple increase of the number of synaptic liberations of acetylcholine and by a shortening of the periods of enzymatic destruction of acetylcholine intervening between repetitive stimuli.

A retardation of the rate of enzymatic destruction of acetylcholine should theoretically have effects similar to those of increased frequency of stimulation. Frequency of stimulation remaining constant, each stimulus should leave a residue of undestroyed acetylcholine proportional to the degree of retardation of destruction of acetylcholine. Repetitive additions of greater residues would accordingly result in greater accumulation of acetylcholine during repetitive stimulation, which would reveal itself in reinforced breathing.

To test this view normal cholinesterase activity was artificially inhibited by administration of several anticholinesterases, and the reflex responses at the several frequencies of stimulation of the carotid nerve compared with control responses obtained before administration of anticholinesterases. Foreign anticholinesterases were injected directly into the vertebral artery in order to confine their inhibiting effects as much as possible to the central nervous system. Figure 2 compares the respiratory response to stimulation of the carotid nerve at given frequencies before and after injection of eserine. It is obvious in figure 2 that temporal summation of stimuli was greater at each frequency of stimulation after the normal rate of enzymatic destruction of acetylcholine had been retarded by anticholinesterase. It may therefore be tentatively concluded that both shortening of the period of physiological destruction of acetylcholine and retardation of the normal rate of destruction of acetylcholine enhance temporal summation of stimuli by favoring greater accumulation of acetylcholine.

Figure 3 illustrates the responses of breathing to repetitive stimulations of the carotid nerve before and after administration of diisopropylfluoro-phosphate. It will be seen that the results are in the main similar to those of eserine recorded in figure 2. Potentiation of temporal summation of stimuli by DFP is particularly striking in the suspension tracings of the facial accessory respiratory contractions.

Figure 4 illustrates results obtained with an unidentified anticholinesterase kindly supplied to us by the Army Chemical Center. This anticholinesterase has been designated as 'X substance'. Unquestionable potentiation of temporal summation of stimuli is again clearly visible in torsal and facial respiratory movements. Note absence of response of facial accessory respiratory muscles to stimulation at all frequencies before administration of anticholinesterase and increasing strength of response with increasing frequency of stimulation after administration of anticholinesterase.

In addition to these experiments on 'foreign' anticholinesterases we have also made a few observations on the effects of carbon dioxide on temporal summation. According to Glick (6) CO_2 possesses anticholinesterase activity varying in intensity with the hydrogen ion concentration it produces. The acid humoral theory of chemi-

cal control of breathing (7) is based on the *in vitro* experiments of Glick which show the greatest changes of anticholinesterase activity to lie within the normal pH range of the body. According to the acid humoral theory of control of respiration, CO₂ functions as an automatic regulator of breathing by grading the effectiveness of prevailing respiratory reflexes. It therefore seemed of interest to compare the magnitude of temporal summation of stimuli at several frequencies before and during artificially produced hypercapnia.

The control records of figure 5 showing respiratory responses to repetitive stimulation of the carotid nerve at frequencies of 10, 25 and 50/second while the dog was breathing pure oxygen from a rebreathing tank may be compared with the lower records showing responses to similar stimulations after the dog has been exposed to a mixture of 10 per cent CO₂ in 30 per cent O₂ and 60 per cent N₂. Note potentiation of the facial accessory as well as of the torsal response to temporal summation during hypercapnia. It is suggested, in agreement with earlier results (7), that CO₂ potentiates the response of the respiratory center to afferent nerve impulses in a manner similar to that produced by foreign anticholinesterases.

Table 1 allows a more quantitative analysis of the data presented in figures 2, 3, 4 and 5. It shows the individual effects of varied frequency of stimulation and of varied rate of destruction of acetylcholine on the reflex response of inspiratory and expiratory muscles. It is interesting to note that potentiation of temporal summation of stimuli by anticholinesterases is in general proportionately greater in the response of the expiratory muscles to sensory stimulation. This generalization may be related to the fact that eupneic breathing is primarily dependent upon the active contractions of the inspiratory muscles. The expiratory muscles contract mainly under conditions demanding greater pulmonary ventilation.

DISCUSSION

Continuing impingement of nerve impulses on a neuron theoretically assures a continuing liberation of free acetylcholine. Presence of cholinesterase theoretically assures a continuing destruction of acetylcholine. The resultant of these opposing tendencies would be expected to maintain a constant basal level of acetylcholine suitable for maintaining eupneic breathing, as represented by the cross-hatched area in figure 6. An increase of acetylcholine above eupneic concentration is assumed to occur in 2 ways: by an increase of impinging nerve impulses and by a retardation of enzymatic destruction of acetylcholine as suggested in the chemical control of breathing by CO₂ (7).

Figure 6 attempts to visualize how these two ways participate in temporal summation of stimuli under the artificial control of frequency of stimulation and of velocity of enzymatic destruction of acetylcholine employed in the present experiments. Each volley of nerve impulses set up by stimuli of uniform strength applied to the carotid nerve is assumed to activate a constant number of synaptic endings and to liberate a uniform amount of acetylcholine. Enzymatic destruction is assumed to follow the law of mass action. Retardation of enzymatic destruction of acetylcholine by administration of anticholinesterases is arbitrarily assumed to lower the velocity of reaction to one-third that of normal.

The schema represents effects of 3 hypothetical frequencies of stimulation of the carotid nerve, 15, 20 and 30 stimuli/second. One hundred arbitrary units of acetylcholine are assumed to be liberated by each stimulus or volley of nerve impulses. Incomplete destruction of acetylcholine between successive volleys leaves acetylcholine residues which accumulate with continuance of stimulation. Increasing concentration of acetylcholine augments the rate and amount of acetylcholine destroyed between volleys in accordance with the law of mass action. Eventually a concentration of acetylcholine is reached at which the amount of acetylcholine destroyed between volleys equals the amount liberated by individual volleys, thus establishing an electrochemical state of equilibrium.

Inspection of the schema shows that the initial acetylcholine residuum left by the first stimulus varies directly as the frequency of stimulation, namely 18, 32 and 45

TABLE 1. CC. INCREASE IN PULMONARY VENTILATION PRODUCED BY REPETITIVE STIMULATION OF CAROTID NERVE

FREQ.		ESERINE		DFP		X-SUBSTANCE		CO ₂	
		Before	After	Before	After	Before	After	Before	After
per second									
10	Insp. Exp.	782	2587 661	962 180	1143 300	842	1384 300	1324	2889
17	Insp. Exp.	1384	3581 1083	1263 240	1745 962	1083	1745 902		
25	Insp. Exp.	2768 240	5235 1985	1865 722	2527 1444	1143	2166 782	1866	3490
33	Insp. Exp.	3310 902	7041 3249	2347 1143	2888 1925	1444	2708 902		
50	Insp. Exp.							1504	3730

arbitrary units at 15, 20 and 30 stimuli/second, under normal conditions before the administration of anticholinesterase. Each second stimulus adds 100 units of acetylcholine to its respective acetylcholine residuum with the result that the peak concentration of acetylcholine rises. The amount of acetylcholine destroyed after *stimulus 2* is greater than that after *stimulus 1*. The absolute rise of acetylcholine concentration from *peak 2* to *peak 3* is consequently reduced. The gradient of acetylcholine accumulation diminishes in this fashion with each added stimulus until a steady state of concentration is reached. This concentration of acetylcholine represented by the horizontal portion of each curve will be seen to vary with the frequency of stimulation, 123, 145 and 178 units for 15, 20 and 30 stimuli/second, respectively.

Retardation of enzymatic destruction of acetylcholine to one-third the normal rate raises the initial acetylcholine residuum from 18 to 57 units at 15 stimuli/second, from 32 to 71 units at 20 stimuli/second and from 45 to 79 at 30 stimuli/second.

Retardation of enzymatic destruction of acetylcholine steepens the initial gradient of acetylcholine accumulation at each frequency of stimulation. Equilibrium is attained later and at a higher concentration of acetylcholine. At a hypothetical frequency of 15 stimuli/second it is 271 units as compared to 123 before artificial reduction of the normal rate of destruction of acetylcholine, an elevation of 148 units. At 20 stimuli/second, it is 318 units as compared to 145 units, an increase of 178 units. At 30 stimuli/second it is 400 as compared to 178 units, or an increase of 222 units.

If one assumes strength of respiratory contractions to be a physiological index of prevailing free acetylcholine in respiratory neurons, it is interesting to compare the theoretical curves of acetylcholine concentrations at hypothetical frequencies of stimulation with the corresponding functional reflex responses of the respiratory muscles. A number of agreements become apparent. 1) The gradient of augmented strength of inspiration is steepest from inspiration No. 1 to inspiration No. 2, as is the corresponding gradient of accumulated acetylcholine. 2) The gradient of augmented strength of inspiration like the gradient of accumulated acetylcholine diminishes with continuance of stimulation. If stimulation of the carotid nerve is sufficiently prolonged, intensity of respiratory contraction reaches a steady state, as illustrated in the lower tracing of figure 1. The fact that a steady state of intensity is reached in both inspiratory and expiratory contractions suggests corresponding steady states of concentration of acetylcholine in the respective half centers. A steady state of strength of respiratory contraction, however, was not reached in figures 2, 3, 4 and 5, because of insufficient duration of stimulation. The closest approximation to a steady state is to be seen in the inspiratory contractions occurring during the highest frequency of stimulation in figure 2, after the administration of eserine. 3) Maximum intensity of respiratory contraction, like maximum concentration of acetylcholine, varied with frequency of repetitive stimulations. 4) Retardation of natural destruction of acetylcholine increased maximum intensity of contraction in a manner similar to the theoretical increase of accumulated acetylcholine. 5) Magnitude of potentiation of reflex response to stimulation of the carotid nerve by anticholinesterase varied with the frequency of stimulation which corresponds with the theoretical equilibrium concentrations of acetylcholine at normal and retarded enzymatic destruction of acetylcholine.

Such remarkable agreement between theory and experimental findings may possibly justify the conclusion that summation is importantly a function of the law of mass action as applied to enzymatic destruction of acetylcholine but it does not preclude participation of other contributing factors. Application of the law of mass action to production of acetylcholine could also be considered. Nor do the present experiments by themselves preclude participation of a peripheral potentiating action of anticholinesterases. Direct and indirect stimulation of striated muscle of the frog, however, show diminution of response of muscle immersed in several anticholinesterases, including eserine (9). Effects of DFP (9, 10), however, suggest that potentiation of temporal summation could be due in part to peripheral action with small dosages. In contrast to the experiments of Lundholm (10), we have found DFP, injected into dogs, produces a hyperpnea which appears to be essentially of central

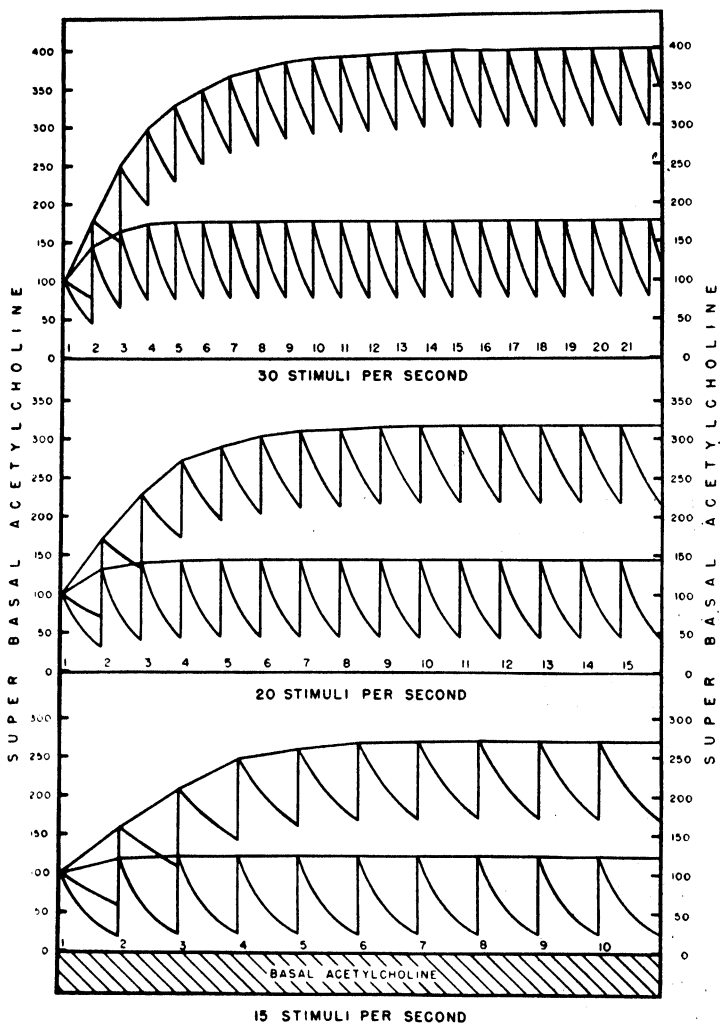


FIG. 6. THEORETICAL ACCUMULATION OF ACETYLCHOLINE in respiratory neurons subjected to repetitive stimulation of respiratory afferents at 15, 20, and 30 stimuli/second, before and after administration of anticholinesterase. Construction of the curves is based on the assumption that each stimulus liberates a uniform amount of acetylcholine, that destruction of acetylcholine follows the law of mass action as applied to enzymatic hydrolysis (8), and that the rate of enzymatic hydrolysis is reduced to one third of normal by administration of anticholinesterase.

origin. Regardless of whether or not peripheral motor potentiation exists, it would be difficult to explain prolongation of after discharge of alternating half-centers, such as seen in figures 2 and 3, in terms of peripheral potentiation alone.

Since cholinergic integration is presumably implemented by electrical energy acting on excitable tissue, it is advisable to recall 4 cardinal factors controlling func-

tional activity of neurons: 1) strength of current; 2) rate of change of strength of current; 3) excitability to current; and 4) change of excitability to current (4). Diminishing excitability to persisting neurocellular currents, namely adaptation, could well be a factor contributing to steady states of contraction occurring late in temporal summation.

Regardless of these and other factors possibly contributing to temporal summation of stimuli, it is believed that rate of destruction of acetylcholine constitutes a most important mechanism of nervous integration. The importance of rate of destruction, however, is held to lie, not in quickness of hydrolysis demanded by the theory of transmission of nerve impulses, but rather in the slowness of destruction required by the theory of electrochemical generation of nerve impulses in the neuron. More specifically the question revolves around continuity of electrochemical energy essential to impulse generation versus discontinuity of electrochemical energy essential to synaptic transmission of nerve impulses.

SUMMARY

Temporal summation of repetitive stimuli applied to the carotid nerve was studied by recording the reflex response of breathing. It was found that temporal summation was augmented by increase in frequency of stimulation and by administration of several anticholinesterases.

Since both procedures theoretically lead to an accumulation of free acetylcholine in respiratory neurons, it was suggested that acetylcholine plays a role in summation of stimuli. Evidence is presented which indicates that the process of summation of stimuli is importantly an expression of the law of mass action as applied to enzymatic destruction of synaptically liberated acetylcholine.

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PONTINE AND MEDULLARY REGULATION OF RESPIRATION IN THE CAT¹

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THE hypothesis that the medullary respiratory center is not inherently periodic, but is dependent upon extramedullary influences for its rhythm, had its inception in the discovery of the Hering-Breuer reflexes and in even earlier accounts of the function of the vagus nerves in respiration. It was not however until Marckwald (1) demonstrated the characteristic inspiratory cramps of vagotomized animals with low decerebrations that the hypothesis gained currency. Today it is perhaps the most widely accepted view of the nature of respiratory periodicity, and the most cogent argument in its favor remains the fundamental observation of Marckwald, essentially confirmed and modified by Lumsden (2, 3), Stella (4, 5), and Pitts, Magoun and Ranson (6).

"Animals which have been decerebrated at a pontine level, though they maintain a normal type of respiration and respond normally to chemical and peripheral nerve stimulation, show a striking response on bilateral section of the vagi. . . . This response consists of a tonic inspiration maintained for seconds to minutes with complete cessation of rhythmic respiration. It may be interrupted occasionally by brief expirations, but unless some form of artificial respiration is maintained the animal soon deteriorates and dies" (6). Lumsden (2, 3) spoke of these prolonged inspiratory spasms as "apneuses" and designated the state as that of "apneusis," meaning "a holding of the breath."

The special significance which has been attached to this phenomenon in the evolution of theories of respiratory rhythmicity centers around the assumption that it represents a basic inspiratory tetanus reflecting the unmodified functioning of the inspiratory center, requiring periodic inhibition from external sources, either via the Hering-Breuer reflex afferents, or a pontine pneumotaxic center, before the normal periodic alternation of inspiration and expiration can be produced. The most recent, and most widely accepted version of this concept, that of Pitts, Magoun and Ranson (6) places the source of the uninterrupted inspiratory drive in the inspiratory center of the medulla. The work of Lumsden (2, 3), however, indicates that the apneustic center cannot be located in the medullary inspiratory center, but that it must be situated more rostrally in the brain stem above the level of the striae acousticae. Although the two theories agree in postulating a fundamentally tetanic inspiratory discharge which must be periodically inhibited by a pontine pneumotaxic center, they differ in the location of the inspiratory center.

Recent experiments on the dog, reported by Nicholson and Hong (7) and by Hoff and Breckenridge (8), suggest that apneusis does not represent an essential component of normal respiration, but rather an exaggerated responsiveness to inspiratory drive arising in supramedullary centers and obscuring a normal periodicity inherent in the medullary respiratory centers. The latter have shown in the dog that 1) apneusis is not permanent, but is periodically suspended to produce expiration at rates which give adequate ventilation in most preparations, 2) apneusis is not total, as evidenced

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by the presence of rhythmic respiratory excursions superimposed on apneusis, and 3) apneusis disappears, with re-emergence of normal breathing, as the preparation deteriorates, or as further transections in the medulla are made, or as cranial nerves entering the brain stem are sectioned.

In view of these findings in the dog, and the complete reversal in the interpretation of apneusis and the origin of respiratory periodicity that they entail, it was considered essential to reexamine the respiratory behavior of the cat upon which much of the preceding work has been done. The results of these investigations indicate that, with minor quantitative differences, the respiratory mechanisms of the cat and the dog are identical, that apneusis is an epiphenomenon not related to the genesis of respiratory periodicity, and that the medullary respiratory mechanism is capable of periodic discharge, even when deprived of vagal and supramedullary influences.

METHODS

This report is based on experiments on 45 cats. The animals were decerebrated at the mid-collicular level by the trephine method under ether anesthesia following bilateral ligation of the carotid arteries and insertion of a tracheal cannula. Hemorrhage from the basilar artery was controlled by momentary digital compression of the vertebral arteries. Gelfoam and clotting globulin were found of value in controlling hemorrhage after transection. In a few experiments, the basilar artery was occluded by the method of Pollock and Davis (9). Vagi were disabled by bilateral section in all experiments. Respiration was recorded by means of tandem accordion-type pneumographs (8).

RESULTS

Nature of Apneusis and Apneustic Breathing. Inspiratory spasm, lasting long enough to be termed apneusis, or interrupted often enough to be termed apneustic breathing, invariably appeared after bilateral vagotomy following brain stem transection within the pons. In 2 experiments, the phenomenon appeared before vagotomy, but in these instances the existence of damage to the vagi had been suspected at the time of carotid ligation. Incidental damage to the vagi may have been the cause of the appearance of apneustic breathing before vagotomy observed by Lumsden (2, 3) and Monnier (10).

After an immediate respiratory stimulation resulting no doubt from the section itself, most preparations passed into a state of apneusis or prolonged inspiratory spasm which lasted in general one to 2 minutes, although longer periods up to 10 minutes were not uncommon. All preparations ultimately exhaled, to end the apneustic cycle, then often took one or more normally phasic breaths and repeated the cycle (fig. 1). Each apneustic cycle was usually progressively shorter than that which preceded it, and ultimately a type of apneustic breathing was established in which cycles of apneusis lasting 10 to 50 seconds were repeated at regular intervals.

Artificial respiration was not given after vagotomy in any of these animals; nevertheless survival as long as 5 hours and 55 minutes was observed. This is in contrast to the observations of Marckwald and of Pitts, Magoun and Ranson that frequent artificial respiration was required to maintain life. Survival times were de-

terminated in 6 other animals in which no further procedures were carried out, and were 1 hour, 43 minutes; 18 minutes; 36 minutes; 9 minutes; 60 minutes; 1 hour,

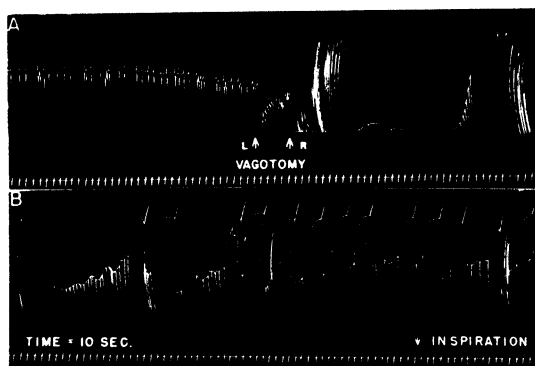
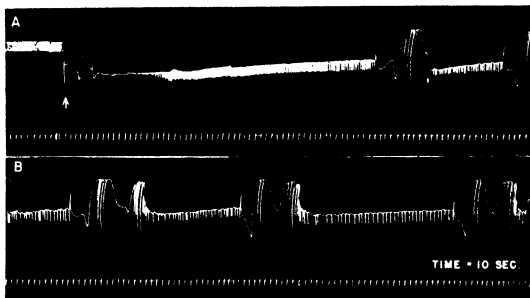


FIG. 1. CAT S 22. *March 2, 1940.* Decerebrated at 1:50 P.M. and after stabilization of respiration was sectioned low in pons at 2:15 P.M. After a period of apnea, spontaneous respiration was reestablished. At 2:45 P.M. the vagi were cut. Subsequent brief respiratory stimulation and diminishing periods of apneusis are clearly observed in A and B which are continuous records. No recordable respiratory motions occurred for the first 2½ minutes of apneusis, but appeared as apneusis diminished at the end of this period. After a series of normal respirations, a second period of apneusis occurred which was shorter in total duration and occluded periodic respiration for only one minute. The third apneustic interval lasted but 20 seconds and had superimposed upon it 4 quite deep respirations. The fourth period of apneusis lasted 1½ minutes and showed significant respiratory excursions. Thereafter the state is best described as one of apneustic breathing, each breath showing one to 4 phasic respirations of low amplitude. This animal succumbed after a third brain stem section. Time in 10-second intervals; inspiration was down in this and subsequent figures.

Fig. 2. CAT S 23. *March 3, 1940.* Mid-collicular decerebration at 3:45 P.M.; midpontine section and vagotomy at 4:07 P.M. Immediate onset of apneusis which lasted 10 minutes. During first minute, apneusis was complete, then more and more marked phasic respiratory movements developed, and provided respiratory excursions almost as great as during control period. Subsequent apneustic intervals were less lengthy and did not completely occlude normal periodicity. At end of each apneustic period, a short series of respirations occurred, followed by a return of apneusis. This animal succumbed during further transection.



50 minutes. In 14 animals other procedures interrupted apneusis, but the average duration of apneusis and apneustic breathing was 45 minutes.

As in the dog, apneusis is not total and does not completely replace normal periodic respiration as has been claimed. There is no doubt that it is more complete than in the dog, and in the first minutes or more may often be attended by total

Fig. 3. CAT S 14. February 22, 1949. After mid-collicular decerebration and a further midpontine section, respiration was stabilized 26 minutes, and vagi were cut. The first cycle of apneusis began $1\frac{1}{2}$ minutes after vagotomy and lasted 1 minute, 10 seconds. Almost all traces of normal respiration disappeared. During next apneustic period, lasting 2 minutes, phasic respirations began. Thereafter 'apneustic breathing' took place for 4 minutes (2 minutes of the record have been removed). A final apneustic interval of 1 minute, 20 seconds then occurred, and thereafter apneusis failed to occur. Records B and C are continuous with record A, and depict a type of Biot's breathing, which, although irregular, shows no traces of apneusis. The animal succumbed 36 minutes after vagotomy.

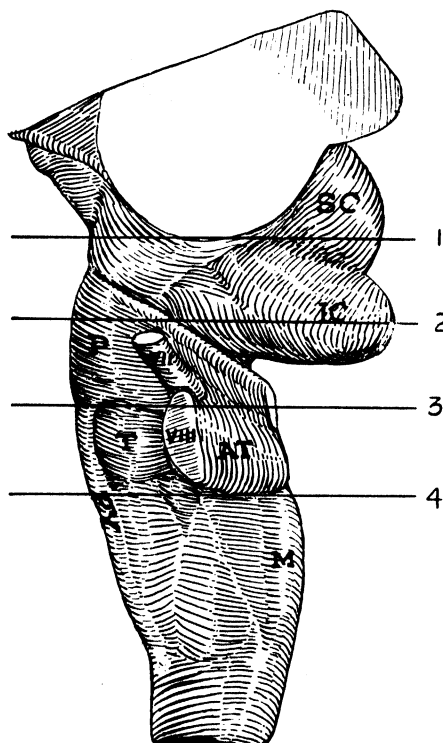
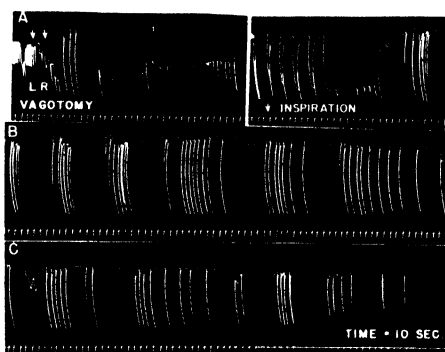


Fig. 4. LATERAL VIEW OF BRAIN STEM of the cat showing levels at which sections were made.

absence of signs of the normal respiratory rhythm (fig. 1). In all periods of apneusis observed in these experiments, however, at times only as an apneustic cycle ended, periodic respiratory movements of the diaphragm, accessory muscles, and even the thorax were observed. They appeared often to be of significant respiratory value (fig. 2). In between long periods of apneusis in the early stages of most experiments, one or more completely normal respirations occurred (figs. 1, 2). Later on, as apneustic breathing developed the apneustic cycles only occasionally consisted of maximum inspirations which were maintained at a smooth tetanic level. More often, one or 2 small phasic inspirations were superimposed, usually at the beginning or the end of even the most pronounced apneustic breaths, and often these phasic components were of significant amplitude, as great or greater in fact than the apneustic component. It can be concluded therefore that in the cat, as well as in the dog (8), apneusis does not entirely replace or

obscure normal periodic respiration. The persistence of these movements, however small, indicates that a mechanism for periodic discharge is retained in these preparations. Only at the extreme height of apneusis are they completely obliterated by

the over-riding inspiratory spasm. Monnier (10), Lumsden (2, 3), and even Marckwald (1) have called attention to this phenomenon in the past, and Lumsden has described the movements as 'gasps,' and indicated that they represent a primitive type of respiratory rhythm bearing no relation to normal breathing. The record shown in figure 2 hardly bears out this assertion, for they are in rate, depth, and respiratory effectiveness surprisingly like normal respirations.

Hoff and Breckenridge (8) have described the return to normal breathing shown by apneustic dogs as the preparation deteriorates. Marckwald (1) had in fact been aware of the same phenomenon. Lumsden also noted the disappearance of apneusis at death and attributed it to the serial dissolution of successively more caudal centers with the progress of asphyxia, which ultimately disabled the apneustic center, leaving

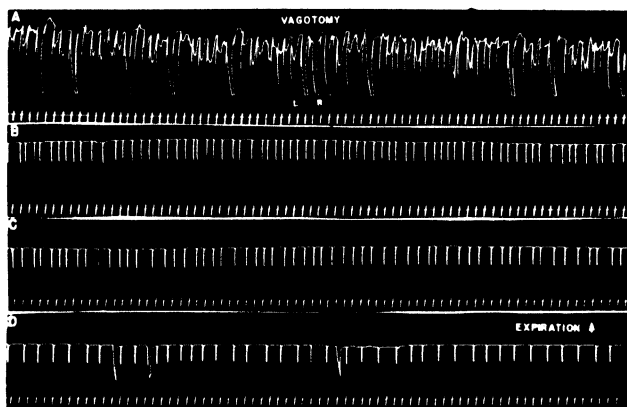


Fig. 5. CAT S 45. April 1, 1940. Basilar artery tied, cerebellum removed and medulla sectioned at level 4. The animal was highly active and the record shows evidences of these phasic spontaneous motions. Vagotomy had no further effect. Shortly after record .1. the brain stem section was squared off, with disappearance of phasic somatic movements. Respiration persisted, however, until the animal was killed. The total survival after medullary section was 2 hours, 50 minutes.

finally a medullary 'gasping' center. This observation is readily confirmed in the cat. As deterioration proceeds, the depth and duration of apneustic breaths diminish while the phasic components grow until finally they alone are visible (fig. 3). Often, as Marckwald observed, apneusis waxes and wanes one or more times before final dissolution.

Levels of Section at Which Apneusis Appears and Disappears. Figure 4 illustrates the levels at which sections were made. In conformity with the reports of Pitts, Magoun and Ranson, apneusis or apneustic breathing followed sections at most pontine levels, i.e. from levels 2 to 3. The apneusis appearing after section at various levels within these limits was not constant, and the most pronounced degrees of inspiratory spasm as regards depth, duration, and degree of occlusion of normal respiratory pattern were obtained following transection in the anterior two-thirds of the pons. Inspiratory spasm which appeared following transection in the posterior one-third of the pons was never as prolonged and was usually

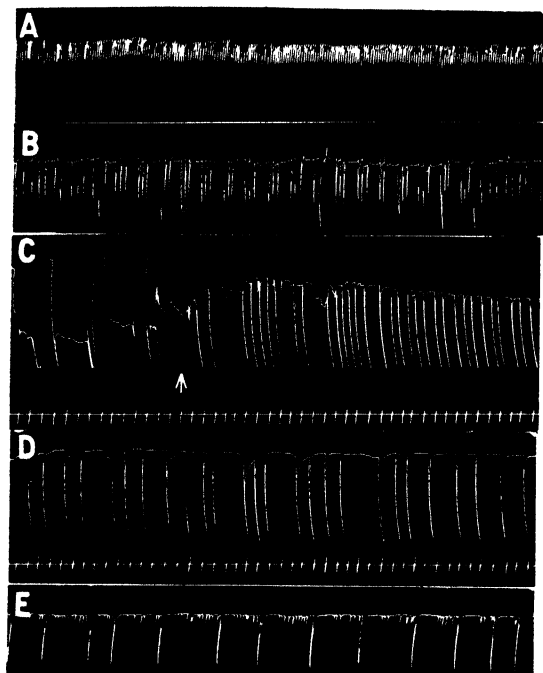


Fig. 6. CAT S 32. *March 21, 1949.* Mid-collicular decerebration at 10:45 A.M. Respiration 32 per minute (record A). Lower pontine transection at 11:12 A.M. (record B). Bilateral vagotomy at 11:35 A.M. Apneustic breathing. Section below the trapezoid bodies at 12:08 P.M. (see arrow, record C), with restoration of phasic breathing of deep all-or-nothing character (records C, D). After approximately 2 hours these respirations became much less frequent, and a series of more shallow breaths at a more rapid rate was interposed. This animal was killed in good condition 2 hours, 22 minutes after the last transection.

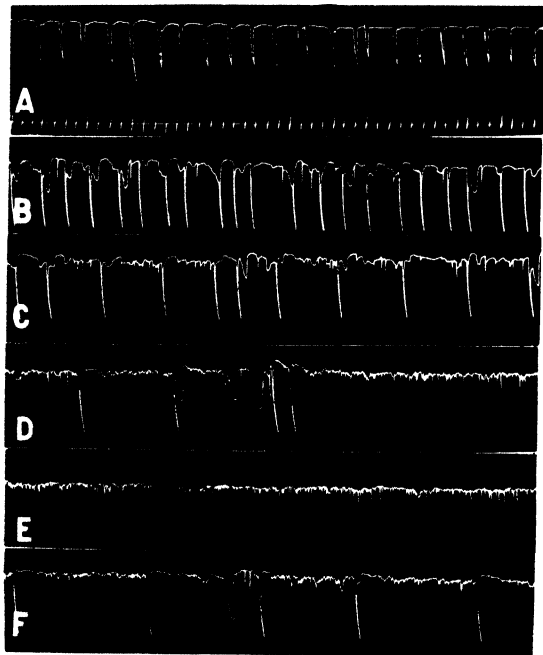


Fig. 7. CAT S 39. *March 25, 1949.* Decerebellation, lower pontine section at 11:50 A.M. (level 3). Respiration deep and slow (record A). Bilateral vagotomy at 12:10 P.M., apneustic breathing not shown. Medullary section (level 4) at 12:20 P.M. with disappearance of apneustic breathing (record B). The deep all-or-nothing breaths became less frequent (record C) and are replaced by small rapid and irregular breaths (records D, E) within the next 3 hours. Later the deep respirations return (record F) and take place about 6 times a minute before death 9 hours after medullary transection. The last vestiges of apneustic breathing may be seen in records B and C, and occasionally in records D and F.

not so complete as with higher sections so that the superimposed phasic respirations were usually much more marked. With more caudal sections within the acoustic tubercles and trapezoid bodies (between sections 3 and 4) apneustic breathing was curtailed, eventually becoming phasic with inspiratory spasms interspersed between normal respirations (fig. 5). Such movements had in these circumstances lost their exclusively respiratory nature and were invariably associated with general bodily movements involving limb and trunk musculature. Decerebrate rigidity had largely disappeared. The movements consisted of motor synergies strikingly recalling athetosis, manifesting themselves in simultaneous and serial involvement of limb and trunk muscles in acts suggestive of twisting, turning, stretching and walking. These involved trunk and proximal joints. Quick rapid movements were seen in the distal joints, such as extension and fanning of the toes in the hind limbs, and running and clawing movements of the front limbs. Movements occurred irrespective of restraint or external stimulation.

With sections high in the medulla, spontaneous motor behavior and associated slight apneustic phases diminished greatly or disappeared completely, and uncomplicated respirations of normal pattern alone remained. As previously reported, many preparations showing minor degrees of spontaneous activity lost it as deterioration proceeded so that in animals surviving for relatively long periods respiration was the sole remaining spontaneous motor activity (fig. 5).

Nature of Medullary Respiration in the Cat. Eighteen animals were included in this portion of the study. The brain stem was sectioned in the upper portion of the medulla either primarily or following higher sections of the brain stem, and both vagi were divided. One animal died immediately, and it was apparent upon examination that the transection was far too low in the medulla to expect continuation of respiration. The remaining 17 animals survived for periods ranging from 16 minutes to 9 hours, with an average survival time of 80 minutes. A number of those surviving for longer periods were killed in order to terminate the experiment, and there is no doubt that if extra precautions had been taken, longer survival might have been recorded.

In almost all preparations a type of respiration was observed which Lumsden (2, 3) described as gasping in character. This corresponded to the all-or-nothing type of respiration Hoff and Breckenridge encountered in medullary preparations in the dog and consisted of maximum respiratory efforts often associated with full employment of accessory respiratory muscles (fig. 6). The rate of these respirations varied greatly. At times it was the sole respiratory act noted and had frequencies in the neighborhood of 4 to 10 per minute; while at other times these breaths were interdigitated with a more rapid shallow type and were much less frequent. The other type of respiration seen in the cat was the more shallow 'ataxic' type also noted by Hoff and Breckenridge in the medullary dog. This respiration was commonly seen and was irregular in rate and depth (fig. 7E). Often, particularly in animals surviving for longer periods, both types of respiration were seen together, the second type forming a more or less continuous background for the less frequent and deeper breaths (fig. 7). Such combinations characterized also many pontine preparations before vagotomy. Biot's breathing appeared in 4 of the 18 animals.

This type of periodic respiration can be distinguished from Cheyne-Stokes respiration by the fact that the individual respirations of each group are of relatively uniform height and the groups do not show the characteristic waxing and waning of amplitude.

DISCUSSION

These experiments confirm in the cat the view of apneusis derived from earlier studies in the dog, that it is neither permanent nor total. It is not a state of continuous inspiratory spasm, and it does not replace all signs of rhythmic respiratory activity. It must then consist of a state superadded upon, or a modification of, the normal respiratory rhythm which it is only at times able to obscure completely.

It is of interest to inquire how the concept of apneusis as a permanent inspiratory spasm has developed in the literature. Partly this appears accountable by the poor state of many animals which therefore succumbed during the first long period of apneusis and did not survive into the period of apneustic breathing. Another factor is almost certainly the technic of temporary vagal blocking which permitted the vagi to be reactivated so that in effect no animals were carried beyond the first stage of apneusis. The probability that in most animals one or both carotids were occluded has also played a part in intensifying and prolonging apneusis (8). Meier and Bucher (11) have recently repeated Marckwald's (1) original experiments in the rabbit, taking care that neither the carotid nor vertebral arteries were even temporarily occluded, and they have failed to find the characteristic permanent inspiratory cramp that has been postulated. As in our experiments, their animals lived for long periods without the assistance of artificial respiration.

It is suggested therefore that the apneustic theory of the genesis of respiratory periodicity be abandoned, and that apneusis be relegated to a position of secondary importance, probably, as suggested earlier (8) as a phenomenon deriving from unequal separation of the medullary respiratory center from the facilitatory and suppressor influences that impinge upon it. Apneusis can thus be equated to a type of decerebrate rigidity of the inspiratory system. However great may be the importance of these factors in the regulation of respiration, and in this light they remain almost entirely for future investigation, they apparently do not have a part in the fundamental processes responsible for the genesis of respiratory periodicity.

The concept then of a permanent inspiratory output of the medulla is an interpretation from facts which on closer inspection favor even more an opposite conclusion: that periodic mechanisms still persist. Pitts has suggested that the remaining rhythmicity depends upon downward projections into the medulla of remnants of the pneumotaxic center, maintaining some feeble periodicity. If this were so, the more caudal the section, the more marked would be the resultant apneusis. Quite to the contrary, the more caudally the section was made in the pons, the less pronounced apneusis became, and it disappeared completely with sections in the medulla. The essential implication of this fact is that inspiratory spasm represents a phenomenon which can be clearly dissociated by appropriate transection from periodic respiration of medullary origin.

These experiments confirm the findings of Lumsden (2, 3) that apneusis disappears after sections below the striae acousticae and corroborate his view that

centers producing apneusis must lie between this level and the upper reaches of the pons. They confirm also the experiments of Nicholson and Hong (7) and of Hoff and Breckenridge (8) in the dog in which apneusis was also demonstrated to be supra-medullary in origin. They fail to afford support for the contention of Pitts, Magoun and Ranson (6) that inspiratory spasm is the product of the medullary inspiratory center.

It should be noted however that Pitts, Magoun and Ranson did not make transections below the lower border of the pons and that if this be taken into consideration in evaluating the theory they have supported, it would be necessary to adopt the view of Lumsden and assume that inspiration is mediated in the normal animal by the action of an apneustic center located in the brain stem at the level of the striae acousticae, and to abandon any idea that the medullary inspiratory or expiratory centers have an essential function in the intact organism. This is in fact the necessary conclusion of Lumsden's hypothesis, and he was led to describe the remaining respiratory acts of the medullary preparation as 'gasps' produced by a primitive medullary gasping center which made no contribution to normal breathing. It is here that our interpretation differs from that of Lumsden, for the respiratory behavior of the medullary preparation did not appear to differ in any basic characteristic from normal breathing, giving a clear impression that the medullary respiratory center, even when isolated from more rostral centers, and after vagotomy, is periodic, and that this periodicity is the fundamental component from which normal breathing is developed. The frequent appearance of normal respirations between apneustic breaths, the failure of apneusis or apneustic breathing to suppress completely periodic respiratory movements, the return of periodic respiration before death, and the restoration of periodic breathing by medullary transection may be considered as strong evidence that the medulla is capable of periodicity. The long survival of many of these preparations suggests that this periodicity is an expression of a normal medullary function and not an unnatural consequence of conditions such as anoxia and trauma.

It seems to be justifiable at present to adopt for the cat the hypothesis derived from study of the respiratory activity of the medullary dog, that respiration in the medullary preparation shows fundamental similarities in rate, depth and rhythmicity with the respiration of the classical mid-collicular preparation before vagotomy. Excepting insofar as it shows a more general lack of regulation appropriate to its more restricted neurological substratum, the respiration of the medullary preparation has appeared to be normal or to constitute the indispensable background for normal respiration.

Although, as has been stated before, the present experiments afford no clues as to the nature of factors generating medullary periodicity, they give promise of simplifying the basic problem by providing a preparation of more restricted anatomical complexity within the confines of which the essential factors are to be sought.

SUMMARY

Apneusis in the cat is not permanent, nor does it supplant completely periodic respirations of a normal type. It diminishes and disappears with return of periodic respirations as deterioration progresses. Apneusis or inspiratory spasm fails to ap-

pear in the vagotomized animal when transections are made below the striae acousticae. Apneusis is considered to be an epiphenomenon arising from supramedullary centers and not intrinsically associated with the genesis of respiration.

The periodic respiration of the vagotomized medullary preparation has fundamental similarities to normal respiration. Respiration is envisaged as a basic periodicity of the medullary centers, regulated secondarily by facilitatory and suppressor areas of the brain stem.

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PHYSIOLOGICAL DETERMINATION OF THE BOUNDARY OF THE ACOUSTIC AREA IN THE CEREBRAL CORTEX OF THE DOG¹

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THE boundary of afferent connections to the acoustic cortex has been needed for the interpretation of electrical activity in the region. The different techniques of neuro-histological investigations have given varying results (1, 2) for the boundary, and have failed to support findings of oscillographic studies (3-5). In addition, silver methods do not permit sufficient differences to be found between afferent connections of sensory systems and the non-specific afferents of adjacent areas (6).

The difficulty of assigning an anatomic boundary with oscillographic methods has been primarily due to the behavior of electric currents in volume conductors. Since the brain is a volume conductor, the action currents of the nerve cells and fibers create electric fields (7-9) which have extensive distributions in the brain medium. With the usual methods of recording, the potentials of the field are obtained at considerable distances from the active neuron. Thus the potential alone cannot be used to indicate the presence of active tissue at the tip of the probe electrode.

In the course of studies on the activity of the acoustic cortex in the presence of intense sound stimuli, an extensive distribution of potentials was found, extending beyond the limits of the accepted afferent area. It was considered important to know whether this distribution of potentials was due to an electric field or to additional afferent connections. A method was found which served to distinguish the parts of the brain showing only electric fields from those containing active afferent fibers. Results of the method are described below for the boundary of the acoustic area.

METHODS

This investigation was performed on 11 dogs. The animal in each experiment was maintained under deep anesthesia by repeated administration of sodium pentobarbital to reduce the spontaneous electrical activity of the cortex. One cerebral hemisphere was completely exposed, and the area surrounding the brain was covered with cotton moistened in Ringer's solution. The dog was placed in a sound-proof room with its head facing a loudspeaker at a distance of one meter from a line through the two ears.

The recording probe electrodes consisted of a linear array of 10 stainless steel suture wires (B & S no. 30), whose tips were spaced 2 mm. apart. The tips of the wires were brought into contact with the surface of the brain by means of a stereotaxic instrument that permitted movement of the array in 2-mm. coordinates over

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the cortex. Each electrode was connected through a separate pre-amplifier to an individual cathode ray tube of a 10-channel cathode ray oscillograph (Tektronix, Inc., Portland, Oregon). The other side of the input circuit for each pre-amplifier was a common point on the moist cotton surrounding the brain. The 10 cathode ray tubes were photographed simultaneously on 35-mm. film which was enlarged on photographic paper to a size satisfactory for analysis. Twenty successive exposures of responses to stimuli were taken for each electrode array setting on a single frame of film.

The stimulus was the rather abrupt onset of a sound, which was produced by an electronic gate synchronized with the sweep of the CR oscillograph. The two general types of sound used were 1) white (or thermal) noise which was employed to stimulate

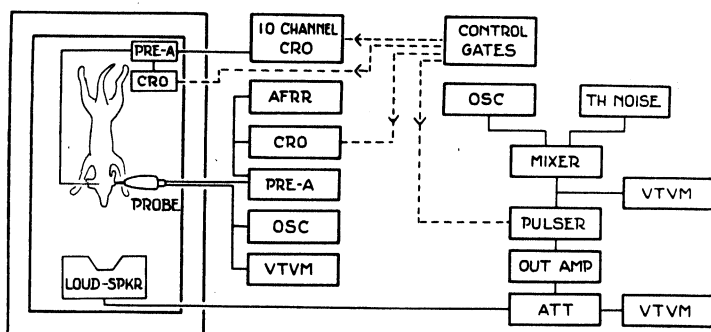


Fig. 1. ARRANGEMENT OF EQUIPMENT FOR EXPERIMENTS. *Left*: sound-proof room with animal in place. Solid line joining units represents signal channels. Dotted line with arrows, pulse lines of control and synchronization gates. *CRO*: cathode ray oscillograph. *PRE-A*: pre-amplifiers. *Pulser*: electronic gate. *OSC*: audio oscillator. *AFRR*: automatic frequency response recorder. *ATT*: attenuator. *Mixer*: signal mixing panel. *TH Noise*: thermal noise generator. *VTVM*: vacuum tube voltmeter or level meter. *OUT AMP*: output amplifier. *Loudspkr*: loudspeaker. *Control Gates*: pulse generator and delayed gates for synchronizing sweeps and sound gate. *Probe*: calibrated condenser transmitter with probe and pre-amplifier.

the entire basilar membrane and 2) intense pure tones which served as supplementary stimuli to test limited regions of the cortex.

Sound pressures were measured at the entrance of the ear canal by means of a probe tube² fastened to a calibrated condenser transmitter. The free field sound intensities were obtained directly from a response curve made with an automatic frequency response recorder, after correction for free field, recording system and output voltage of the probe microphone. The intensity levels of the sound pulses are given as decibels above the standard reference level of 10^{-16} watts per cm^2 for the steady state condition. The schematic of figure 1 shows the general arrangement of the equipment for each experiment.

Three per cent strychnine sulfate solution colored with toluidine blue was applied locally to minute areas of the cortex with a 1×2 mm. piece of filter paper. The paper was allowed to remain in contact with the brain for a few seconds and

² The probe tube was kindly placed at the author's disposal by W. A. Munson of the Bell Telephone Laboratories.

was then removed. The toluidine blue indicated if spread of the solution occurred over the cortex apart from the area immediately under the paper strip. Frequent spontaneous strychnine spikes at the electrode nearest to the applied drug served to indicate that a high concentration of the strychnine was present within the cortex. Induced strychnine spikes to the sounds were taken as evidence for the existence of afferent connections to the point of application of the drug. If no change of the potential occurred, it was concluded that no afferent connections existed. After records had been taken, to avoid delay in waiting for the natural disappearance of the strychnine action, the spikes were suppressed by placing a large piece of filter

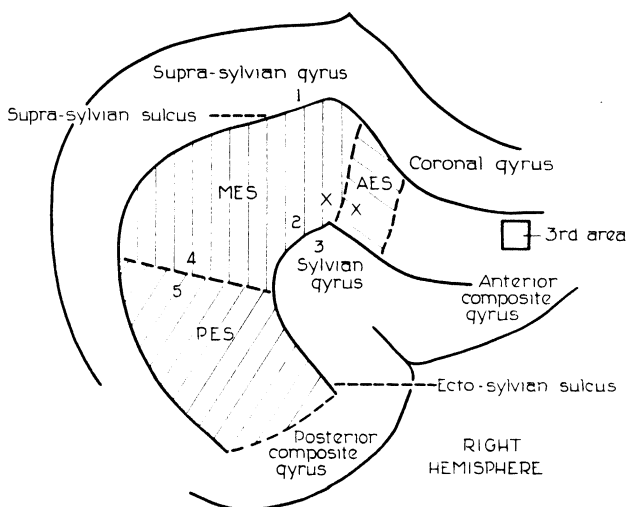


Fig. 2. Shaded: area from which induced spikes were obtained to sound stimuli. Subdivided by hatching into anterior ecto-sylvian (AES), middle ecto-sylvian (MES) and posterior ecto-sylvian areas (PES). Numbers and X's refer to examples of figs. 3 and 4.

paper soaked in 6 per cent sodium pentobarbital over the areas trychninized. Thus, large areas of the cortex could be examined rapidly, virtually titrating the strychnine action with sodium pentobarbital.

RESULTS

Intense sounds produced responses over wide-spread areas of the cerebral cortex, including the ecto-sylvian, supra-sylvian, coronal, and sylvian gyri (fig. 2). The activity centered on the middle ecto-sylvian gyrus and diminished in directions away from this area. The dorsal boundary of the acoustic area was located by systematically applying the small pieces of paper with strychnine over the supra-sylvian and coronal gyri until induced strychnine spikes were found. Each application was tested with thermal noise and pure tones. No induced strychnine spikes were obtained anywhere in this region unless the paper or the solution crossed the supra-sylvian sulcus. A typical response from the supra-sylvian gyrus is shown in figure 4 1a. Failure of

strychnine to alter the potential is illustrated by *1b*. Thus, the dorsal boundary of the afferent connections is represented by the supra-sylvian sulcus.

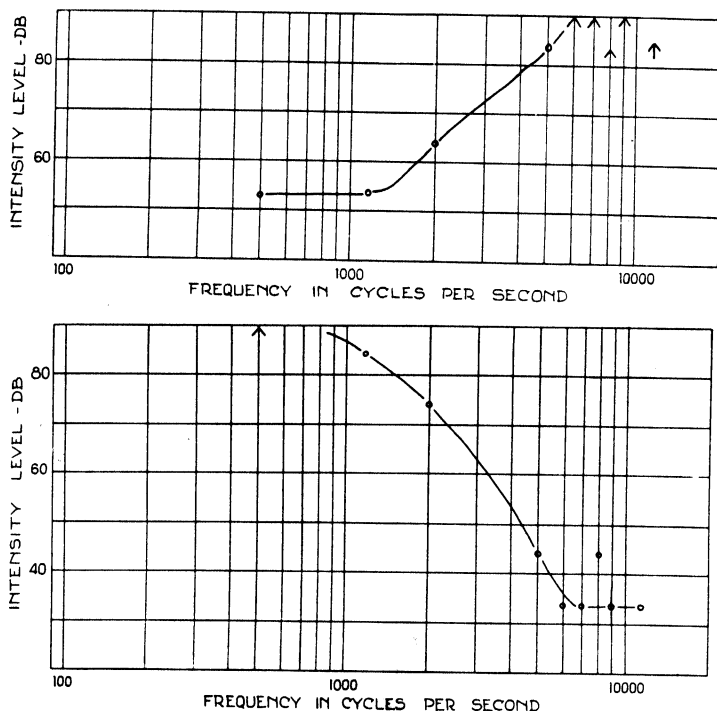


Fig. 3: *A* (upper). THRESHOLD CURVE for strychnine spikes at X (fig. 2) of anterior ecto-sylvian area (AES). *B* (lower). Threshold curve for spikes at X (fig. 2) of middle ecto-sylvian area (MES). Arrows indicate level at which threshold was not attained.

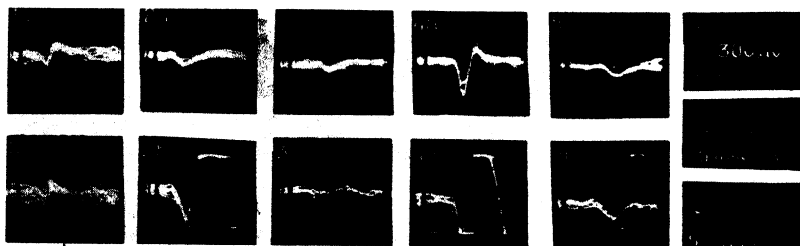


Fig. 4: EXAMPLES OF RESPONSES to local strychnine from cortical points corresponding to numbers in fig. 2. 1. 4000 cps. at 83.8 db. 2, 3: 2000 cps. at 79.8 db. 4, 5: 400 cps. at 83.8 db. *a* Response before strychnine. *b*. After strychnine. Flattening of spike peaks due to blocking of amplifiers. Upward deflection: surface negative.

The sylvian gyrus has always possessed small responses of the type illustrated in figure 4 *3a*. Although both thermal noise and pure tones elicited responses in this region, tones of the middle frequency range most readily produce them. Local applica-

tions of strychnine to the sylvian gyrus have failed to yield induced spikes (3*b*). Since the sylvian and ecto-sylvian gyri are generally separated by the ecto-sylvian sulcus, it appeared possible for the mid-frequency region of the ventral area to be hidden in the sulcus. One dog, lacking a sulcus between the middle ecto-sylvian and sylvian gyri, was studied with this in view. No strychnine spikes to sounds were found on the sylvian gyrus in this case, but activity was encountered dorsal to a line which joined the ends of the anterior and posterior ecto-sylvian sulci. The spikes (2*a*, 2*b* of fig. 4) above this line were readily obtained at intensity levels characteristic of the dorsal area. Furthermore, there was a spatial arrangement of the potentials—responses to high frequencies found anteriorly, to low frequencies, posteriorly. No reverse pattern was found.

On the posterior ecto-sylvian gyrus the activity of strychnine on the potentials was present ventrally to the junction of the posterior ecto-sylvian with the composite gyrus.

The entire ecto-sylvian gyrus was found to give strychnine spikes to sound with the exception of a region between the dorsal part of the anterior ecto-sylvian and the third area (fig. 2). The cortex surrounding the third area was examined with strychnine, but no spikes were found. Strychnine applied to the third area caused spikes to noise and all test tones. Thus, no continuity existed between the third area and the other portions of active cortex, although occasionally potentials were found distributed between them.

The ecto-sylvian gyrus has been grossly studied with systematic applications of strychnine. By this method it has been possible to divide the gyrus into three major areas (AES, MES, and PES of fig. 2). The anterior ecto-sylvian area (AES) was sharply separated from the middle ecto-sylvian by a boundary that was determined by taking the threshold curves for the induced strychnine spikes (figs. 3 *A* and *B*). Thus far, only low frequencies have caused strychnine responses in the AES. Parenthetically, it should be stated that the electric fields of the potentials alone for low and high frequencies overlapped so extensively in this area, that an examination of the threshold of these potentials gave an erroneous impression of the boundary.

The boundary between the middle and posterior ecto-sylvian gyri was determined by examination of the induced potentials. In the MES region, the induced spikes had short latencies, a constant threshold, and showed a definite localization pattern for different frequencies. Also, the responses occurred to almost 100 per cent of successive stimuli (fig. 4 *4a*, *b*). On the PES area, the spikes were delayed in their onset, the threshold varied over 40 to 50 db, no relation to the localization of the MES was observed, and the responses appeared to about 50 per cent of the successive stimuli. Characteristic responses are illustrated in figure 4, *5a*, *b*.

DISCUSSION

The acoustic area of the dog, determined by the strychnine method described in this paper, agrees essentially with the cyto-architectonic cortex, ecto-sylvian B of Campbell (10), and with the results of the oscillographic study reported earlier (4) at threshold values. In the latter report small potentials found on the sylvian gyrus and erroneously attributed to the ventral area can now be disregarded as electric

field potentials. The reason for their appearance between the anterior and posterior ecto-sylvian gyri was due to the higher intensities required for demonstrating the so-called ventral area. These higher intensities caused a marked spread of the electric field from the adjoining mid frequency region of the dorsal area over the sylvian gyrus, thus filling the gap between the extremities of the ventral area.

The absence of strychnine spikes on the sylvian gyrus raises doubt as to the organization of the ventral area or *area II* in the pattern originally described by others (5). Failure to locate the mid frequency portion of such an area in these experiments is due either to an anomaly of the acoustic system or to the fact that the region still remains hidden within a sulcus. The differences in the characteristics of the responses in the anterior ecto-sylvian (AES) and posterior ecto-sylvian (PES) areas also suggest that they belong in two distinct systems rather than a common one.

The boundary of the dorsal area is in agreement with the outline of the active region found by Bremer (3) for the rhythmic activity due to continuous tones in the encephalole isole preparation. Results to 'click' stimulation reported by various authors cannot be compared, since no information is available on the spectral characteristics of the click stimulus.

Strychnine has been used successfully for investigations of cortico-cortical and various subcortical connections in the nervous system. Its specific usefulness in the study of afferent systems of the cortex in general is implied by the following features of its action on the acoustic system: 1) Spontaneous strychnine spikes indicate its presence in sufficient concentration. 2) Sensitivity: the induced spikes are obtained at intensity levels of 0 to -10 db for the optimum spectral range, therefore at threshold levels probably the minimum number of fibers are involved (cf. threshold of human hearing). 3) Discrimination: by examining the cut-off frequencies, a band as narrow as 1/10 octave is readily measured at 10,000 cycles per second, which is equivalent to 0.2 mm. of cortical surface. 4) Locally applied sodium pentobarbital suppresses its action rapidly, permitting re-application without excessive delay. 5) The band-pass characteristics of a strychninized point in the cortex agree more closely with the data for human hearing (11, 12) and the nature of the information from the cochlear nucleus (13) than do the characteristics of the non-strychninized point.

The method confirms the existence of the third acoustic area.

SUMMARY

A physiological method has been described for determination of the boundary of the acoustic area in the cerebral cortex of the dog. The method consisted of applying strychnine locally to the cortex and stimulating with sound. The appearance of an induced strychnine spike was used to indicate the existence of an afferent connection. The acoustic area was bounded by the supra-sylvian and ecto-sylvian sulci. Anteriorly, the boundary was a line across the dorsal end of the anterior ecto-sylvian gyrus. Ventrally, the area was limited by the junction of the posterior ecto-sylvian and composite gyri.

The middle ecto-sylvian gyrus was characterized by a specific arrangement of the responses with respect to frequencies, lowest thresholds, and consistency of

responses. This area has been termed tentatively the primary projection area. On the dorsal end of the anterior ecto-sylvian gyrus, there exists a small distinct area for low frequencies, not activated at usual levels by high frequency tones. It does not extend across the sylvian gyrus. The posterior ecto-sylvian gyrus is distinct physiologically from both this anterior area as well as the area of the middle ecto-sylvian gyrus.

The third area is a 2 x 2 mm. region isolated from the remainder of the acoustic cortex.

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THERMOSENSITIVITY OF THE TURTLE BRAIN AS MANIFESTED BY BLOOD PRESSURE CHANGES

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WE have recently called attention to a relationship between body temperature and arterial pressure which can be observed in many vertebrates including poikilotherms as well as homeotherms. When the body temperature of these animals is lowered, a concomitant fall in blood pressure is seen; with rewarming a stepwise return to the original pressure levels occurs.

Abundant data in the literature support the concept that this is a general vertebrate response to changes in body temperature. Comparative biological data show that birds, which have a higher body temperature than mammals, also have a higher blood pressure (1). The association between temperature and pressure has been demonstrated among the cold-blooded species in the frog (2), the turtle (3, 4) and in the alligator (2). Among mammals, inspection of published data shows the thermobaric covariance in man (5), dog (6-8), cat (9), rabbit (10) and in hibernating animals (11). It has also been demonstrated in birds including the pigeon (12) and the chick (13). In some mammals, such as the rat, the blood pressure may be maintained at near normal levels despite the induction of hypothermia, possibly because of the elicitation of homeostatic mechanisms such as shivering which act to maintain a constant body temperature (14). However, even in these animals, if cooling is marked, a blood pressure fall which is reversible in rewarming is seen. This is borne out by the fact that newborn rats which have not yet developed temperature-regulating mechanisms show a thermobaric relationship (15).

Because the relationship appears to be so general among vertebrate species, analysis of the mechanism whereby the blood pressure is associated with the body temperature would appear to be of value. The covariance apparently does not depend upon coincidental changes in heart rate, cardiac output (8) or circulation time (16) which occur with marked changes in body temperature, nor can it be accounted for on the basis of variations in the reactivity of the blood vessels (13, 16, 17). This is shown by the fact that there are no significant changes in the pressor response to epinephrine (13, 17) or to the depressor action of acetylcholine (16) in hypothermia or fever. It does not depend upon the presence of functioning adrenal glands (9).

The thermobaric relationship appears to depend upon the intermediation of the central nervous system, because section of the cervical spinal cord or destruction of the brain results in the elimination of the expected changes in pressure when the body temperature is varied (17). This neurogenic mechanism may operate via stimulation of specific receptors in the periphery which act through the central nervous system, or it may depend upon direct stimulation of the neuraxis itself. If blood pressure changes resulted from direct thermal stimulation of the brain without concomitant change in body temperature, the results could be considered as evidence for the presence of a temperature sensitive center in the brain.

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METHODS

Blood pressures were recorded on 44 turtles (*Pseudemys elegans*), by removing a circular plate 3 inches in diameter from the plastron, exposing the left aorta, and introducing a cannula directed peripherad into this vessel (4). In this way the pressure in the right aorta is measured since the left and right aortae join below the transverse septum to form the dorsal aorta beyond the point of cannulation. The cannula was attached to an optical recording Hamilton manometer (18), and blood pressure records were made on photosensitive paper.

The head was held firmly away from the shell by means of forceps attached to the lower jaw. A trephine hole 4 mm. in diameter was made in the skull over the portion of the brain to be warmed or cooled. In some experiments a second trephine hole was made for the insertion of a thermocouple for the purpose of determining the conduction of heat through the brain. A mercury thermometer was inserted into the rectum for the measurement of body temperature.

The apparatus used for cooling and warming the brain consisted of the barrel portion of a 100-cc. syringe containing a coil of silver wire, about 1.5 mm. in diameter, one end of which passed through the lumen of the needle adapter and extended one half inch beyond the tip. The tip of the wire was pushed through the brain substance until it came to rest against the floor of the cranial cavity. After the wire was inserted into the brain, it was not moved for the duration of the entire experiment. With this method, heat was conducted to the brain from the warm or cold water in the barrel of the syringe. The wire was alternately cooled and warmed for periods of 5 to 10 minutes each. The temperature of the animal, as indicated by a mercury thermometer in the rectum, remained unchanged during the experiment. The location of the wire in the brain was checked by gross examination at the time of the insertion of the wire and at the end of the experiment. At the end of each experiment, the head was removed, placed in formalin and later examined grossly to determine the region in which the wire had been placed. This was facilitated by dipping the silver wire in india ink just prior to insertion into the brain, thus marking the path of insertion.

RESULTS

Effect of Implantation of the Wire into the Brain. Insertion of the silver wire into the brain was usually accompanied immediately by a marked rise of 3 to 17 mm. Hg (average 10) which often persisted for the duration of the experiment. This led to generally higher values for blood pressures than we observed in earlier experiments on the turtle (4). This increase in blood pressure was accompanied by changes in heart rate, with either a slowing or an acceleration. The rise in systolic pressure was on the average greater (12 mm. Hg) than the rise in diastolic (8 mm. Hg). The rise in pressure appeared to occur regardless of the site of implantation of the wire. A similar rise in pressure was often seen on the insertion of the mercury thermometer into the rectum, although this rise in pressure did not persist.

Estimation of the Thermal Stimulus. To estimate the degree of thermal stimulation in the present experiments, measurements were made of the transmission of heat from the silver wire to a thermocouple in contact or at a distance of one millimeter from the silver wire. The intervening medium was air, dead turtle brain or

the brain of an intact living turtle. Equilibrium was reached at about 2 minutes after adding the warmed or cooled water to the reservoir. Table 1 gives results obtained in this series of experiments during the period immediately following the addition of water to the syringe barrel.

It can be seen that transmission of heat from the syringe water to the brain had a low efficiency. On the basis of these findings it is likely that the thermal stimulus applied to the brain was less than 10°C . at the tip of the wire. At points one millimeter away the temperature differential was considerably less. The role of transmission of heat by the circulating blood is indicated by the lower temperatures obtained in the living brain as compared with those obtained when using dead brain.

Effect of Thermal Stimulation. The response to warming or cooling the brain on the blood pressure was tested in 368 trials on 44 turtles. A typical response to warm-

TABLE 1. TRANSMISSION OF HEAT FROM WIRE TO THERMOCOUPLE ACROSS DEAD OR LIVING TURTLE BRAIN

MEDIUM	DISTANCE OF THER- MOCOUPLE FROM WIRE	TEMPERA- TURE OF ADDED WATER	TEMPERATURE OF THE BRAIN				
			Before	½ min.	2 min.	4 min.	8 min.
				after changing temperature of water in syringe			
Dead brain	mm.	°C.	°C.				
	0	80	30	38	41	42	42
		4	28	22	21	19	18
	1	80	27	30	32	33	33
Living brain	0	80	26	32	35	36	36
		5	27	25	21	20	19
	1	76	26	28	31	31	31
		6	26	25	23	22	22

ing and cooling is illustrated in figure 1 and the protocol of a typical +++ experiment is given in table 2.

A. Warming. The effect of various degrees of local warming was studied in 191 trials. When the thermal stimulus was of low grade, as when the water in the syringe was only 10 to 20°C . above that of the turtle, no consistent changes in blood pressure were seen. When the temperature differential was great, as when the water in the syringe was up to 50°C . above the temperature of the turtle, the blood pressure generally increased, although the rise was dependent upon the site of the implantation of the wire. Intermediate degrees of warming resulted in moderate increases in pressure².

When a thermal stimulus was applied, the blood pressure usually began to rise in about 20 seconds and the rise continued, reaching a maximum in 3 to 4 minutes. Following the pressor effect, the pressure began to return to normal levels as the water in the syringe cooled toward room temperature. No significant or consistent changes in heart rate were seen during the blood pressure change. The blood pressure

² It is unlikely that the responses obtained are due to tissue injury because the temperature change in the brain was much less than in the water in the syringe (table 1) and furthermore because the response on cooling was reversible and was followed again by a pressor response on subsequent rewarming.

rise varied from 3 to 10 mm. Hg, depending, as noted, upon the intensity of the thermal stimulus and the site in the brain in which the wire was placed.

B. Cooling. Local cooling of the brain was tested in 177 trials. As with the warming experiments, the degree of blood pressure response depended upon the intensity and site of the stimulus. When the water placed in the syringe was only 8 to 10° C. below that of the body temperature of the animal, no consistent changes in pressure were seen. When the water was 15 to 22° C. below that of the animal, a fall in pressure of as much as 10 mm. Hg was sometimes seen, depending upon the site of insertion of the wire.

The most pronounced changes in pressure were seen when the reservoir water was changed immediately from warm to cold or vice versa, thus increasing the intensity of the thermal stimulus acting at the site of the wire.

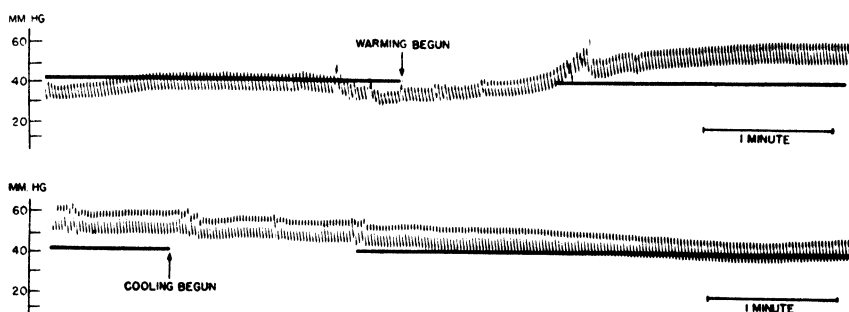


FIG. 1. OPTICAL RECORDING of arterial blood pressure changes induced by warming and cooling of the turtle brain. At "Warming begun," water at 75°C was added to the syringe, resulting in conduction of heat via a silver wire to the brain. At "Cooling begun," warm water was withdrawn, and cold water at a temperature of 5°C introduced. This produced cooling of the brain at the site of insertion of the needle. In this experiment, the wire was inserted in the midline at the level indicated by the line leading from the notation "cerebral hemisphere" in figure 2. (See text.)

Effect of Site of Stimulation. It soon became apparent that not all regions of the brain were equally responsive to thermal stimulation, as manifested by blood pressure changes. It therefore became necessary to explore various regions of the brain to determine the local thermosensitivity. In a series of 29 turtles the effect of marked thermal stimulation was measured (fig. 2). In these experiments, the water used was either 75° or 5° C. for the warm and cold stimulus, respectively. It was found that the most thermosensitive level was at the level of the midportion of cerebrum as noted in figure 2. This portion of the cerebrum overlies the third ventricle and its adnexa. Experiments in which the wire was placed in the olfactory lobes or medulla gave equivocal results in that usually no consistent blood pressure changes were seen on repeated stimulation with heat or cold. Similar inconsistent responses were seen when the needle was in the optic lobes, the cerebellum and the medulla (fig. 2).

The responses were graded according to the consistency with which repeated warm or cold application produced blood pressure increases and decreases, respectively. Thus experiments in which warmth produced an early and marked increase in pressure in all trials, while the alternate cooling trials produced a marked fall in blood pressure, were noted as ++++. When no significant or consistent blood

pressure changes were noted with either cooling or warming, a grade of 0 was recorded. Slight to moderate responses were graded as +, ++, or +++ depending upon the criteria noted. In 2 experiments in which the wire was at the level of the cerebrum, warming consistently produced a fall in blood pressure while cooling produced a rise. These sites are recorded as *R* (reversed response) in figure 2. An experiment with the wire in the region of the third ventricle classified as a +++ response is summarized in table 2.

DISCUSSION

Although it has long been known that warming or cooling of the warm-blooded brain can lead to a variety of changes, particularly those related to regulation of the body temperature (19, 20), the present experiments demonstrate for the first time the existence of a temperature-sensitive center in the brain of a cold-blooded

TABLE 2. TYPICAL EXPERIMENT SHOWING BLOOD PRESSURE RESPONSE TO THERMAL STIMULATION OF THE BRAIN¹

	COOL		WARM		COOL		WARM		COOL		WARM		COOL		WARM		COOL		WARM	
	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D
Control.....	25	15	24	15	25	15	25	15	32	22	25	16	30	20	25	15	25	15	22	15
1 min.....	0	0	+2	+1	0	0	+5	+4	-2	-2	+3	+3	0	0	0	+1	-1	0	+1	0
2 min.....	-3	-3	+4	+3	-3	-2	+7	+5	-7	-7	+5	+4	-4	-2	+5	+4	-2	-1	+6	+3
3 min.....	-3	-3	+6	+5	-5	-5	+5	+4	-8	-7	+5	+4	-8	-6	+5	+3	-3	-3	+5	+3
4 min.....	-3	-2	+4	+2	-5	-5	+7	+5	-7	-7	0	-1	-4	-4	0	+1	-3	-2	+4	+1
5 min.....	-3	-1	+2	+3	-5	-5	+7	+5	-7	-6	+7	+6	-5	-5	0	0	-3	0	+3	0
10 min.....	-1	0	+6	+3	0	0	+7	+7	-7	-6	+7	+6	-5	-5	0	0	-2	-1	+3	0

Cooling stimulus: water placed in syringe was 5°C.; warming stimulus: water placed in syringe was 70°C. *S* = Systolic and *D* = Diastolic pressure. Control line gives the value in millimeters of mercury immediately before application of stimulus. Time values indicate duration of the thermal stimulation. Values given in the columns below the control value show the change from control values.

¹ Site of wire implantation was region of third ventricle, June 16, 1949.

animal. Warming of this region of the brain by our technique resulted in an immediate rise in arterial pressure, while cooling resulted consistently in an immediate fall in pressure. Comparison of our data on the rate of warming or cooling of the intact turtle brain (table 1) with the rate of blood pressure change following thermal stimulation (table 2) shows a striking similarity in the time course of events. In the absence of significant heart rate changes, the observed blood pressure variations must be attributed to neurogenically controlled variations in the calibre of the arterioles, capillaries and venules with a consequent increased resistance to the flow of blood through the peripheral vessels.

The finding of a temperature-sensitive region in the brain of a cold-blooded animal which effects blood pressure changes raises the question of its possible physiological function. The functions probably are related to the adaptation of the poikilotherm to its constantly changing thermal environment. Poikilotherms are at the mercy of variations in their environmental temperatures since they have no mechanisms for maintaining a constant temperature. During the course of an hour or two on the land, their body temperatures may vary as much as 25° C. The great

changes in the metabolic rate occurring as a consequence of such temperature variations, together with that caused by prolonged activity may be adjusted by changes in the internal environment. Thus, the increased blood pressure and blood sugar (21) seen on increasing the body temperature of cooled animals may be adaptive mechanisms for supplying an increased blood flow and augmented nutritive materials to the more rapidly metabolizing cells. These thermobaric and thermoglycemic responses are both controlled through the central nervous system (22), possibly via thermosensitive centers. Other changes in the internal environment in response to body temperature variations, such as shifts of water from the blood to the cells (22) may also be neurogenically controlled.

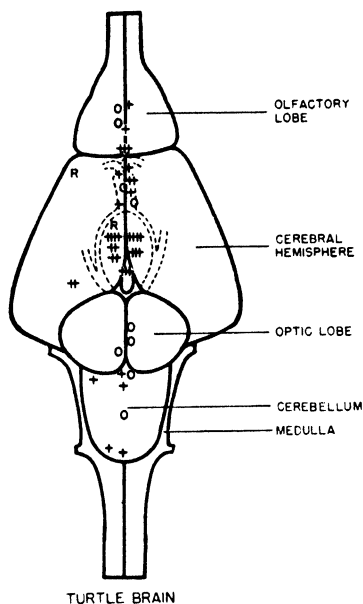


FIG. 2. OUTLINE OF THE TURTLE BRAIN with notations indicating intensity and consistency of blood pressure changes following thermal stimulation of the brain at the sites noted. The dotted lines represent optic nerves, chiasm and tracts and serve to indicate the region of the third ventricle. In this region the most marked and consistent responses, indicated by +++ or ++++ were seen. Lesser degrees of response are indicated by + or ++. Those sites at which thermal stimulation produced no consistent responses are shown as O. In 2 instances warming produced a fall in blood pressure while cooling produced a rise. These sites of implantation of the wire are given as R (reversed response). (See text.)

The localization experiments show that the most thermosensitive region of the brain is in the midline near the midportion of the cerebrum, at the level of third ventricle (fig. 2). No attempt at vertical localization was made. The wire was pushed through the substance of the brain until it came to rest upon the inner table of the floor of the cranium. Therefore in this site the wire was in contact with the floor of the third ventricle. It is of considerable interest to note that the region of greatest thermal sensitivity in a cold-blooded reptile, the turtle, is the same region as that in which the temperature-regulating apparatus has evolved independently in both the mammals and the birds. Convergent evolution in this case suggests that a pre-condition for the development of temperature regulation was already resident in the hypothalamus of the cold-blooded progenitors of the present homeotherms. Such a thermosensitive center capable of making adjustments in the internal environment of the poikilotherm may have formed the anatomical basis for the later evolution of temperature-regulating mechanisms.

The positive responses seen at sites outside of the region of the third ventricle may have been caused by the vascular transmission of heat from the site of the insertion of the wire to more sensitive centers or possibly by a diffuse distribution of the thermosensitive cells. However, the pressor response to insertion of the wire into the brain probably depends upon mechanisms different from those involved in the response to thermal stimulation. This interpretation is supported by the fact that insertion of the wire into the brain or of a thermometer into the rectum produce changes in heart rate and in reflex activity of the animal, as indicated by running movements, in addition to blood pressure changes. On the other hand, introduction of the thermal stimulus to the brain resulted in no change in heart rate and in no outward expression of activity of the animal. Therefore the thermobaric phenomena probably act enteroceptively, directly on the calibre of the smaller peripheral blood vessels without eliciting the somatic responses ordinarily seen with exteroceptive stimuli.

SUMMARY

A temperature-sensitive center, manifested by thermally induced blood pressure changes, is present in the brain of the turtle, a cold-blooded animal. Direct warming of the turtle brain results in a rise in blood pressure, while cooling results in a fall in pressure. The degree of the response is dependent upon the intensity of the thermal stimulus and upon the site of stimulation. The most sensitive site was found to be at the level of the third ventricle. The significance of these results in terms of physiological adjustment of the poikilotherm to environmental temperature changes, and in terms of the evolution of homeotherms is discussed.

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PRESSOR SUBSTANCES IN ARTERIAL HYPERTENSION: ACTIVITY AND AMINE CONTENT OF CRUDE EXTRACTS OF BLOOD¹

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A SEARCH for pressor substances has been conducted for many years both in human beings and in experimental animals (1). Usually these experiments resulted in failure to demonstrate any positive effect. In dogs, however, Solandt, Nassim and Cowan (2) were able to demonstrate some effect by transfusing blood from hypertensive to nephrectomized animals. Freeman (3) reported that the hypertensive dog's blood differed from normal blood when transfused. In the blood of human beings with so-called 'essential hypertension,' it is undecided, however, whether or not vasoconstrictor substances exist, although the weight of evidence is in favor of their presence. And yet, Host (4), Pickering (5) and Prinzmetal and his colleagues (6) were unable to demonstrate any effect of hypertensive venous blood on the blood pressure of normal subjects in cross transfusion experiments, even if large quantities were used.

To know whether or not pressor substances are present in blood of cases of arterial hypertension in man is of the utmost importance. A series of experiments was begun (7), therefore, in an attempt to demonstrate their presence. In preparing extracts, the following assumptions were made: *a*) that arterial blood contains more of the hypothetical pressor substances than does venous blood. This assumption was based on the possibility that the kidneys or other organs with direct venous return to the heart might elaborate these substances and that they would be metabolized in part during passage of blood through the arterioles, just as happens in the case of epinephrine; *b*) that the substances are present in very small amounts; *c*) that they are unstable, being easily oxidized or inactivated perhaps by enzymes in the blood; *d*) that possibly they are more or less complex amines.

This paper reports the progress made in this search prior to interruption by the war. Evidence is presented on the presence of pressor substances. It appears that, when acidified, these are soluble in 90 per cent alcohol and insoluble in petroleum ether and in ether.

METHODS

Preparation of Blood Fractions. Arterial blood was drawn directly into an Erlenmeyer flask containing an amount of 95 per cent alcohol equal to 2 to 3 times

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the volume of blood (i.e. 200 cc. of blood to 400–600 cc. of alcohol) (fig. 1). The amount of alcohol was small because the amount of blood to be drawn was not known. Later, alcohol was added to give a total of 10 volumes of alcohol. The precipitate was removed by filtration. Concentrated HCl was added to the filtrate (one cc. for each 200 cc. of blood). The filtrate was concentrated (usually overnight) to dryness *in vacuo* at 30 to 35°C. The dry residue was then extracted briefly several times with a minimum amount of alcohol, a volume approximately equal to the original amount of blood. It was centrifuged and the precipitate discarded. The supernatant fluid was freed of alcohol by vacuum distillation and the residue was extracted with petroleum ether.

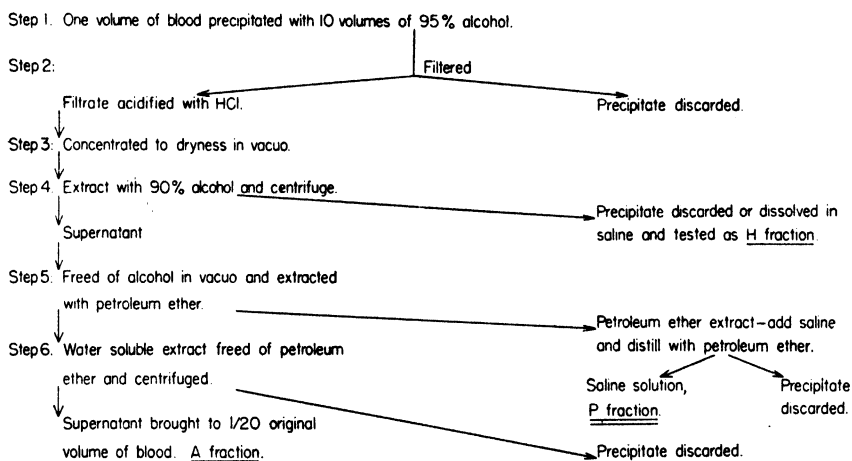


Fig. 1. PREPARATION OF BLOOD EXTRACTS. The pressor activity of the various fractions of hypertensive blood in rats was as follows: *H* fraction, 1 of 4 injections; *P* fraction, 7 of 17, with 3 giving only immediate pressor effects; *A* fraction, 29 of 41, with 4 giving immediate responses (see text). Extracts of normotensive blood were active as follows: *P* fraction, 2 of 9 injections; *A* fraction, 4 of 25, with one immediate response.

The petroleum ether extract was discarded after preliminary tests with saline extracts of it showed that little or no pressor material was present. The petroleum ether was removed by vacuum distillation. The residual aqueous layer was centrifuged to separate any insoluble material. If the volume of the aqueous residue was small, saline was added before centrifugation to $\frac{1}{20}$ the volume of the original blood. This preparation was labeled the *A* fraction. Other fractions are shown in figure 2 and in some instances are discussed later in the text. Before injection into rats to test the presence of pressor activity, the *pH* of the extracts was adjusted to approximately 7.4 with sodium bicarbonate or sodium hydroxide solution.

Measurement of Amines. For the purpose of estimating the relative concentrations of amines in the preparations from blood, a modification of Richter's method (8) was employed. To one cc. of material, 4 cc. of 2 *M* NaOH and 6 cc. of toluene were added. The mixture was shaken well for 5 minutes and centrifuged whenever necessary to separate the 2 layers. After the extraction, 4 cc. of the toluene extract were

added to 4 cc. of an 0.08 per cent solution of picric acid in chloroform in a dry tube. The intensity of the yellow color formed was estimated in terms of the color developed by various amounts of isoamylamine extracted in the same manner. It was recognized that the procedure would not give precise information on the amine content because 1) not all amines are extracted (for example, the hydroxy phenylamines), 2) the simple amines do not give a color, and 3) the various amine picrates have different transmission spectra. In some instances the amines formed picrates that crystallized. These were collected and tested for their pressor activity.

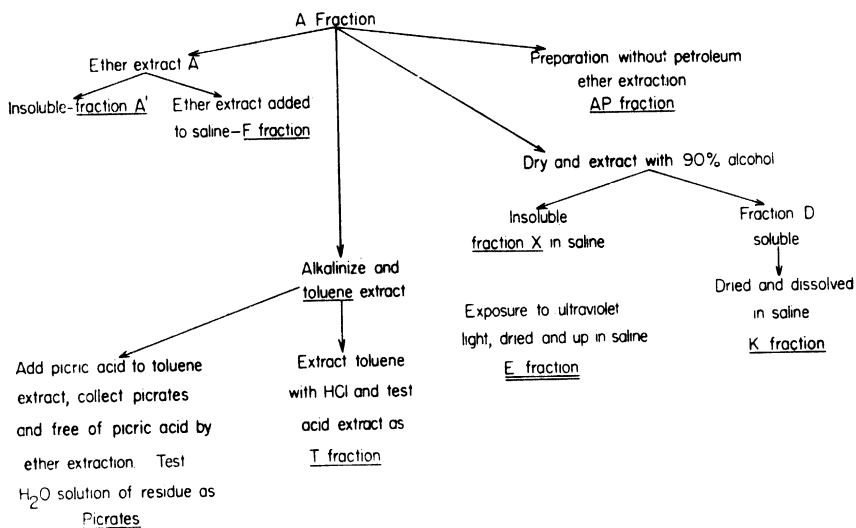


Fig. 2. MODIFICATIONS OF A FRACTION gave positive prolonged pressor effects in rats as follows: Hypertensive. *A'*, 2 of 6 injections; *F*, none of 5; *AP*, 3 of 8; *X*, 2 of 7; *K*, 2 of 5; *E*, 3 of 18, with 8 others giving immediate pressor responses; *T*, 2 of 4; and Picrates, 9 of 13, with one immediate response. Normotensive. *X*, none of one injection; *E*, 3 of 10; *T*, 1 of 3; and Picrates, 1 of 5.

Tests for Pressor Activity. Preparations were tested in rats for their pressor activity. Rats weighing 200 to 350 gm. were anesthetized with sodium pentobarbital given intraperitoneally. Tracheotomy was performed because, when this anesthetic was used, rats often died of upper respiratory obstruction. The femoral vein was isolated and a blunt needle tied into it. The femoral artery on the opposite side was isolated. A few crystals of novocaine were placed on it followed by 2 or 3 drops of physiological saline solution to prevent spasm of the femoral artery, so as to permit the insertion of a 22- or 23-gauge needle connected with a Hamilton manometer. An adequate amount of heparin was then injected intravenously. Approximately 160 rats were used. One cc. of the extract, which corresponded to 20 cc. of original blood, brought to a pH of 7.4, was injected. The effects on the blood pressure were recorded on a photokymograph. An elevation of the diastolic pressure of more than 12 mm. Hg, 5 to 20 minutes after injection, was considered to be significant.

Sixty-five samples of human arterial blood taken from 23 patients suffering

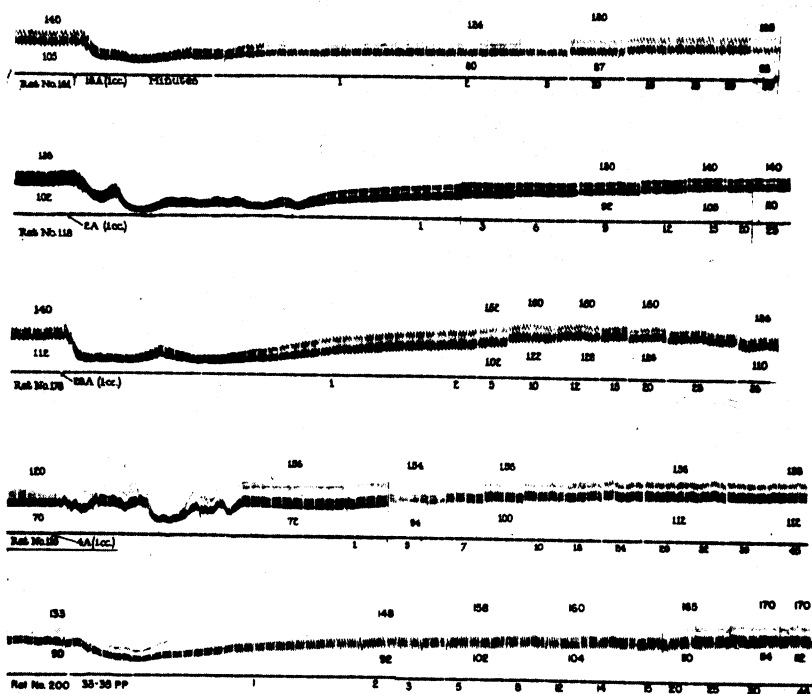


Fig. 3. TYPICAL RESPONSES OF RATS' BLOOD PRESSURE to injections of extracts of blood. The blood pressure is indicated by the curve photographed from the Hamilton manometer. The figures above refer to the systolic, below to the diastolic pressure in mm. Hg. The time is recorded on the lower line at 2-second intervals, the numbers indicating minutes after injection. *Rat 161* was injected with extract 18A from a normal subject. There was an initial depression (considered +++) not followed by a rise. *Rat 118* was similarly injected with extract 2A from a patient exhibiting congestive heart failure. The initial depression (considered +++) was not followed by a significant rise. *Rat 178* was injected with extract 28A from a patient with arterial hypertension. The initial depression (+++) was followed by a typical delayed pressor response reaching its height of 160/128, an elevation of 20/16, in 12 minutes and gradually returning to normal levels after 25 minutes. *Rat 135* was similarly given extract 4A from a hypertensive patient. The depressor response in this case was of short duration (+) and was followed by a rise in blood pressure which lasted 45 minutes or longer. Note the initial widening of the pulse pressure. *Rat 200* was injected with mixed picrates (one cc.) and showed an initial depression (considered +) followed by a significant rise lasting 30 minutes.

from arterial hypertension and from 22 normal subjects were extracted. Blood from 2 normal dogs and from one made hypertensive by wrapping the kidneys in cellophane was also tested. The amounts of the samples were usually 200 to 500 cc. Smaller amounts were also taken for special studies.

RESULTS

Types of Response. Certain preparations exerted a pressor effect in rats. Two types of response were noted. One resembled that seen after injections of familiar

pressor substances, in that it occurred immediately, was over in from 2 to 5 minutes and was associated with widening of the pulse pressure. This was characteristic of several groups of extracts.

The second type, usually preceded by a depressor effect, became obvious 2 to 5 minutes after injection. It frequently lasted 20 minutes or longer (fig. 3). This response differed from that seen after injections of renin, angiotonin, epinephrine, tryptamine or isoamylamine, in that it was more prolonged. But it did resemble slightly the effect which follows the injection of tyramine, and even more that produced by phenylethylamine. Mixtures of active extracts of blood incubated with preparations of amine oxidase were inactive.

The effect of injections of extracts from hypertensive and from normal individuals differed. Pressor effects (in rats) were caused by a majority of those from hypertensive patients, and by only a minority from normal individuals.

Results According to Types of Subjects. a) Hypertensive patients. Material which raised the blood pressure in rats was found in a majority of the extracts made from the blood of hypertensive patients. In all, 46 extracts from 21 of the 23 patients showed pressor effects. These were demonstrated in samples taken at different times in 7 cases and in single samples in 13 (table 1). On the other hand, 30 samples were without effect. For example, 8 injections from 5 samples of arterial blood from the same patient were consistently inactive. Extracts from another case were active only in one of 4 injections from 2 samples. Extracts made from the blood of 3 patients yielded no pressor response.

In patients exhibiting renal diseases or involvement, the results presented a different picture (9). In 16 patients with little or no diminution of renal function, 39 extracts were active and 11 inactive.

Two samples of blood from another patient were active. A radical sympathectomy was performed and his blood pressure returned to normal. A sample of blood, taken several months after operation, possessed no pressor activity (fig. 4). In 5 hypertensive patients, classified as 'neurogenic,' extracts from 3 possessed little active pressor principle while that of one possessed none. In one case the results were doubtful. Only 7 of 26 injections of various extracts of blood from this type of case showed pressor substances in the rat. The blood of one patient, in whom pheochromocytoma was found at autopsy, was inactive.

b) Normal subjects. The extracts of blood from 4 subjects with normal blood pressures showed evidence of pressor substances. One of these subjects later developed arterial hypertension and another was suffering from congestive heart failure. There was no activity in extracts made from 18 others. Thirty-two injections of various types of extracts were inactive; 5 were active. Four samples of blood taken at different times from 1 subject and 2 from another showed no activity. In only one case were 2 injections of extracts active.

Results According to Types of Extracts. Fraction A. Pressor effects were demonstrated in 26 preparations of 31 different bloods from hypertensive patients, and only in 4 from 14 normal individuals. Of those suffering from renal hypertension, 29 of 31 injections were active, from neurogenic or other types, none of 10. This fraction therefore appeared to contain material giving more consistent results (fig. 4). Other fractions than A were studied to a smaller extent (figs. 5, 6).

The method of fractionation is presented in figure 1. The main fractions are emphasized there. Losses occurred during the fractionation. In several instances, when the first alcoholic filtrates were kept overnight in the cold before concentration, a slight precipitate formed. This material, collected in the cold and dissolved in saline, caused no characteristic pressor activity. A few tests seemed to indicate that

TABLE 1. PROLONGED PRESSOR ACTIVITY OF VARIOUS ACTIVE AND PARTLY ACTIVE FRACTIONS, AND AMINE CONTENT OF EXTRACTS OF ARTERIAL BLOOD (FRACTIONS A, A', AP AND PICRATES)¹ FROM HYPERTENSIVE AND NORMOTENSIVE SUBJECTS

PATIENT NO.	SEX	NO. BLOOD SAMPLES	EXTRACTS TESTED		AMINE CONTENT λ/cc. ²	REMARKS
			Pos.	Neg.		
Renal Hypertension—Severe						
7	♂	2	3	2 ³	> 10	1 Post-operative
8	♀	1	2	0	3	
9	♂	1	2	0	3	
12	♂	2	4	0	0	Post-operative
15	♀	2	1	2		
17	♂	1	0	1		
18	♂	2	3	0	> 10	
19	♀	1	1	1	1.5	
21	♂	1	2	1	9	Post-operative Heart failure
22	♀	1	2	0	> 10	
27	♂	1	1	1	> 10	
Renal Hypertension—Moderate						
4	♂	3	4	1 ³	3.5	1 Post-operative
13	♀	1	1	1	9	
28	♂	3	5	2	3.5	
29	♂	1	1	0	4.5	
31	♂	1	3	0	9	
32	♂	2	4	1	5	
Total.....	17	26	39	11		
Per cent positive....	94		78			
Neurogenic Hypertension—Severe						
1	♀	2	1	3	> 10	
5	♂	5	0	8	> 10	
Neurogenic Hypertension—Moderate						
16	♂	1	1	1		Pheochromocytoma
25	♂	2	4	2	> 10	
26	♂	1	1	3	> 10	
20	♂	1	0	2	3.5	
Total.....	6	12	7	19		
Per cent positive.....	67		27			

TABLE I.—*Continued*

PATIENT NO.	SEX	NO. BLOOD SAMPLES	EXTRACTS TESTED		AMINE CONTENT γ/cc. ³	REMARKS
			Pos.	Neg.		
<i>Normotensive Patients</i>						
2	♂	1	0	2	1	R.H.D. Heart failure
3	♂	2	0	3	1	A.H.D. Heart failure
6	♂	1	1	0	1	R.H.D. Heart failure
10	♀	1	0	1	0	A.H.D. Heart failure
11	♂	1	0	4	0	A.H.D. Heart failure
23	♀	1	0	1	1	Post-nephrectomy
34	♂	1	0	2	3.5	Glomerulonephritis
Totals	7	8	1	13		
<i>Normotensive Normal Subjects</i>						
14	♀	1	0	2	0	
21	♂	1	0	1		
24	♂	1	0	3	4	
30	♂	4	0	2	1.5	
33	♂	1	2	0	4	
35	♀	1	1	0	6.7	
36	♂	2	1	1	4	
37	♂	1	0	1		
38	♀	1	0	1		
39	♂	1	0	2		
40	♂	1	0	1		
41	♀	1	0	1		
42	♂	1	0	1		
43	♂	1	0	1		
44	♂	1	0	2		
Totals	15	19	4	19		
Per cent positive	18		13			

NOTE: Severe hypertension = diastolic pressure usually at levels of 130 mm. Hg or above. Moderate hypertension = diastolic pressure usually between 110 and 130 mm. Hg.

NOTE: Per cent positive: The first figure refers to the number of cases from which one or more extracts were positive. The second figure refers to the number of positive extracts.

¹See figures 1 and 2. ²In terms of isoamyl amine of original blood. ³One sample taken after lumbo-dorsal sympathectomy.

there was little loss of activity at *Step 4*. This solution showed little or no pressor activity (*H* fraction). Pressor activity appeared in the *P* fractions. This represented material extracted by petroleum ether but which remained in solution in saline when the petroleum ether solution was freed of solvent in the presence of saline. It is possible that other material extracted by petroleum ether may have facilitated removal of the active material. The characteristic activity did not appear to be associated with substances soluble in ether (*F* fraction). It apparently was lost when care was not taken to free the ether of peroxides.

When it appeared that active materials could be obtained from many hypertensive samples but few from normal ones, an attempt was made to discover whether or not the presence of pressor activity could be detected by means of a characteristic ultraviolet absorption spectra. For this purpose the *A* preparations (one cc.) were dried and re-extracted with 90 per cent alcohol to give the *D* fractions. In a few instances the insoluble residue taken up in saline (*K* fraction) gave pressor effects as did the *X* fractions. While it appeared early that there might be a correlation between the presence of an absorption band at 2900 Å and pressor activity, additional samples made it evident that this was not the case. It was noticed, however, that the *D* fractions used for the ultraviolet absorption, when dried, redissolved in saline (*E* fraction) and injected into rats frequently gave a type of pressor response different from that originally present (fig. 5). It appeared to be more like that of the simpler amines. It is not clear whether or not the change resulted from additional

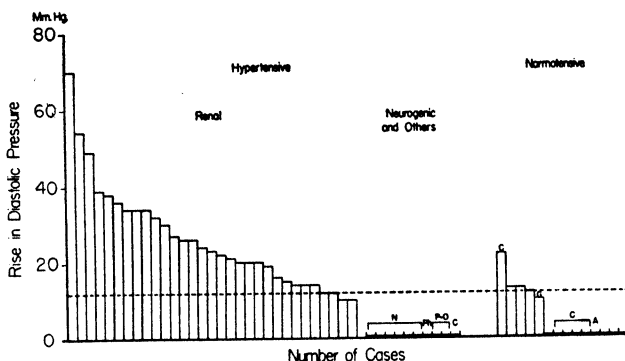


Fig. 4. PRESSOR EFFECTS OF *A* fractions of arterial blood. Bars indicate sustained rise of diastolic pressure of rats measured 5 to 30 minutes after injection of an extract (usually at 15 minutes). All injections are shown. Dotted line represents a change of 12 mm. Hg, which is considered significant. *N* refers to neurogenic hypertension, *PH* to pheochromocytoma, *P.O* to post-operative, *C* to congestive heart failure, *G* to glomerulonephritis, and *A* to arteriosclerosis (see text).

handling of the material or depended specifically on the brief exposure to ultraviolet radiation. A modification in the activity of tyramine after exposure to ultraviolet is recalled by this experience (10), hydroxy-tyramine apparently being formed.

A pressor activity at first similar to that of the *E* fractions but later sustained was observed in the picrates obtained in the estimation of amines. The picrates were collected, acidified, and freed of picric acid by extraction with peroxide-free ether. The resulting solutions showed some pressor activity in 9 of 12 samples from 11 hypertensive patients and in one of 5 from 4 normal individuals (fig. 6).

Miscellaneous Observations. Although preparations were made, as a rule from arterial blood, pressor activity was obtained from venous blood in at least one instance. In several instances, amine picrates were formed. Very few experiments were made with separated components of blood. In some instances, the *A* fraction prepared from plasma was active; in others, it was obtained from cells of partially clotted blood. Adequate data on the distribution of pressor substances in blood are, however, lacking because whole blood was usually drawn directly into alcohol.

a) *Depressor effects.* Many of the extracts contained depressor material which may have masked some pressor activity. There was no correlation between primary depressor effects and subsequent appearance of pressor responses. Some of the fractions relatively free of depressor activity were the picrates and the *P* and *E* fractions. The depressor material was insoluble in acetone.

b) *Chromatographic adsorption.* It was possible to effect some purification of the pressor extracts through absorption of the *A* and *D* fractions on an aluminum oxide column. The fractions were dried and dissolved in 10 per cent methyl alcohol-90 per cent acetone for adsorption. The major portion of the active material was eluted with mixtures of methyl alcohol and acetone, when the methyl alcohol content

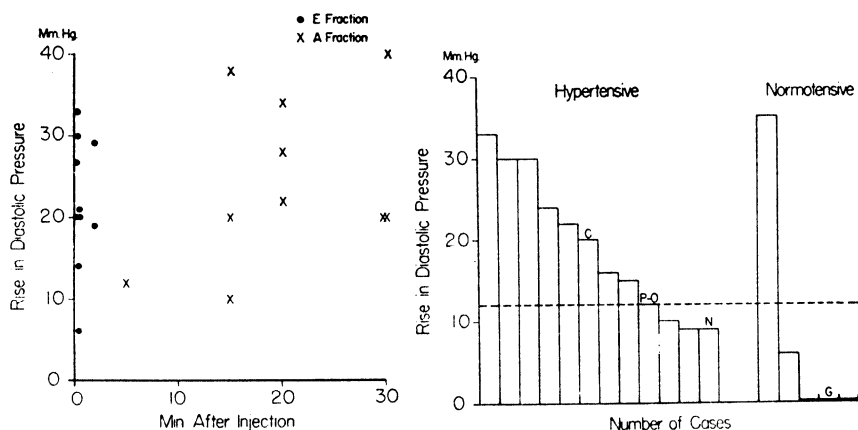


Fig. 5. *Left.* CHANGE OF TIME OF PRESSOR RESPONSE of extracts exposed to ultraviolet light (*E* fraction). The crosses represent *A* fractions, the dots *E* fractions made from the same samples. The height of the activity of the latter occurred usually less than one minute after injection, and was soon over, as contrasted to the prolonged pressor response to the *A* fractions. All samples were from hypertensive patients.

Fig. 6. *Right.* PRESSOR EFFECTS of picrates made from extracts of arterial blood. Notations same as figure 4.

was between 20 and 50 per cent. Lack of material prevented more extensive use of this technique. It was not possible to compare the adsorption and elution of these fractions with angiotonin, for the sample of this substance available was found to contain a number of active pressor components.

c) *Effects of amine oxidase on the activity.* A number of the *A* fractions incubated in the Warburg apparatus with amine oxidase⁴ showed a small absorption of oxygen. Afterward the material was no longer pressor. Material incubated under the same conditions without the enzyme retained its pressor effect. Likewise amine oxidase appeared to inactivate angiotonin and to cause a small absorption of oxygen. Similar tests with tyrosinase and *A* fractions of blood showed some loss in pressor activity though further loss might have been obscured by the presence of pressor material

⁴ We wish to express our appreciation to Dr. A. Walti of Merck & Co., Inc., for supplying amine oxidase.

in the enzyme preparation. Similar amounts of oxygen were utilized by *A* fractions from normal and hypertensive patients.

Effects of the Injection of Pure Compounds. Thirty-five injections of various substances were made in order to learn whether or not pressor responses similar to those seen after use of the active extracts of the blood could be produced. Similar results were not observed after the injection of angiotonin, tryptamine, isoamylamine, epinine, isovaleraldehyde, acetone soluble and insoluble fractions of an angiotonin preparation, casein hydrolysate, epinephrine and tyramine. One substance, phenylethylamine, did occasion a typical response in 4 of 7 instances. The addition of 100 γ of tryptamine to 2 *A* fractions resulted, after injection, in a state of shock, although the extracts contained little depressor material, and tryptamine was found to be a pressor substance.

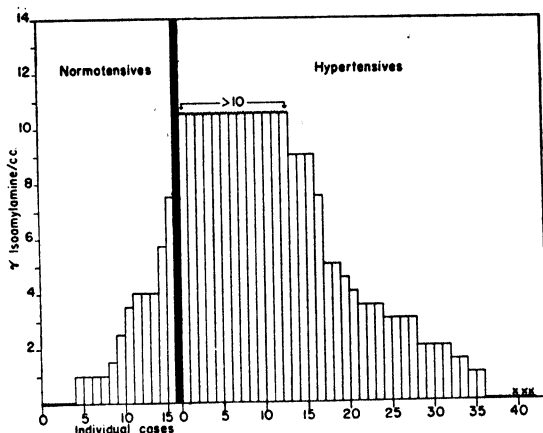


Fig. 7. COMPARISON OF AMINE PICRATES, measured in terms of isoamylamine, in normal and hypertensive blood extracts. All extracts in which their color was estimated are included. The amount indicated is in terms of γ /cc. of original blood. The broken line indicates values which were too high to be read without dilution with the colorimeter at hand, that is more than 10 γ /cc. The samples marked X were those from which extraction was made with ether-containing peroxides.

Correlation Between Pressor Response and Intensity of Picrate Color. There was only a fair correlation between the presence of pressor material and the amounts of amine picrate (table 1). It was not good, possibly because the method of estimation did not measure all of the amines. Figure 7 shows diagrammatically the relative amounts of amine picrates present in the normal and hypertensive groups. It can be seen that the latter group tended to be higher.

Dogs' Blood. Blood extracted from 2 normal dogs was inactive. One sample of 4 taken from a dog, made hypertensive, was inactive, but in 2 others an immediate pressor response was noticed.

DISCUSSION

It appears from the results described that when blood from hypertensive patients is extracted, according to the method outlined in this report, preparations capable of raising the blood pressure of normal rats are usually obtained. We conclude that pressor substances were present in the blood of most of the cases of arterial hypertension which were studied. It appears also that normal blood extracts were, for the most part, inactive.

It is noteworthy that the blood of over half of the hypertensive patients classified as 'neurogenic' yielded no active pressor material. This observation suggests either that pressor substances are present only intermittently and in small amounts or that some other mechanism underlies their hypertension. Further evidence in favor of this possibility has been gained (11). In patients with renal involvement or disease, the differences found are evidence that hypertension is a manifestation of a group of diseases.

The estimation of amines in extracts of blood suggested further that there might be more in hypertensive than in normal individuals. It should be emphasized that severe limitations in the method of estimation exist: 1) phenolic amines such as tyramine and epinephrine are not extracted in the procedure; 2) smaller amines such as the methyl amines do not produce a color; and 3) the color formed is not an accurate index of amine content as different amine picrates exhibit different transmission spectra. The picrates have accordingly not been considered accurate measures either of the total amines or of the pressor substances present in the blood or the extract. The method was used to learn whether or not an estimate of the relative amounts of measurable amines would indicate an underlying metabolic disturbance in the deamination of amino acids.

Evidence on the nature of the pressor substances in the obviously crude extracts is incomplete. From the preliminary observations it would appear that when acidified they are soluble in 90 per cent alcohol and insoluble in petroleum ether and in ether, though possibly inactivated by ether which contains peroxides. When alkalinized, some of the pressor material is extractable with toluene and forms picrates, from which pressor activity may be recovered.

Information gained from the method of preparation of the *A* fractions, from the estimation of amines and recovery of activity from the picrates, suggests that the pressor activity found in the majority of the cases of hypertension in this study is due to more or less complex amines. It is anticipated that further studies now in progress will elucidate this idea.

The methods used in this study should receive comment and criticism. Anesthetized rats were found to make good test animals, as long as anoxia from respiratory obstruction did not occur. The sensitivity of these animals to the active material as compared with other animals or preparations was not tested. It may be that the sensitivity of rats can be increased by some procedure. At any rate, their blood pressure remains remarkably constant for relatively long periods of time, tending only to become lower. We have not noticed spontaneous elevation of blood pressure after the level has become established. The amount of fluid injected or the content of salts or other materials arising from preparing the materials probably did not affect the results. This belief is fortified by controlled observations with many other blood extracts, with hypertonic (6%) saline, with *A* extract containing 9 per cent alcohol and with relatively large amounts and volumes of various materials not reported here.

It has been suggested (12) that renin may have been liberated by the kidneys of these patients when relatively large volumes of arterial blood were removed and that the results may have resulted from the formation of angiotonin or hypertensin.

We do not believe that this substance or its products account for the results observed. The pressor response of the active extracts was in no way similar to that seen after injection of angiotonin.

It is of considerable importance to discover the chemical nature of this pressor material. These preliminary observations merely point the way toward further study of this and similar substances; they may play a decided part in the mechanism of some forms of arterial hypertension.

SUMMARY

Arterial blood from hypertensive and normal patients was extracted and concentrated. The extracts were then injected into rats for the purpose of discovering the presence of pressor substances. In a majority of the extracts from 21 of 23 hypertensive patients, prolonged pressor effects were found. One fraction especially contained the active material. In a majority of the extracts from 22 normal individuals these pressor effects were absent. The amine content of the extracts of hypertensive blood was usually higher than those of normotensive blood.

This study offers evidence that blood from hypertensive patients contains prolonged pressor substances which are not present in most normal individuals. Possibly the substances are amines.

It is a pleasure to acknowledge the technical assistance of Miss Elizabeth Van Pelt. We are indebted to Dr. Konrad Dobriner for performing the spectrographic analyses and for his advice and criticism and to Dr. Alfred E. Cohn for his aid in the preparation of the manuscript.

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INFLUENCE OF THE LIVER ON VASCULAR REACTIVITY

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THE pressor and depressor responsiveness of the cardiovascular system is an integral part of the problem of arterial hypertension. The extent to which vessels constrict or dilate and the myocardium is stimulated or depressed is dependent not only on nervous stimuli, or on the presence and concentration of humoral substances, but also on the responsiveness or reactivity of the vascular components. It is this responsiveness as it results in a rise or a fall of arterial pressure and the mechanisms which alter it that are the subject of the study reported.

There are a few terms which should be defined. Tachyphylaxis is the phenomenon of decreasing responses during repeated injections of some vasoactive substance. Such a lack of response is usually specific to the one drug injected although related substances may also be involved. Refractoriness differs in that it is not dependent on prior injections of a drug. It may come on spontaneously or during a variety of changes in the internal and external environment of the animal. For example, large doses of BAL and Benadryl, or terminal shock from any cause, elicit refractoriness. Such lack of response is not limited to a few vasoactive substances or stimuli but covers a wide range. When responsiveness, pressor or depressor, is increased as a result of denervation or injection of certain drugs, the broad term, augmentation, is preferred somewhat to the term, sensitization.

In this study, responsiveness, or 'vascular reactivity,' as measured by changes in arterial pressure, was determined for a variety of substances: the animal was subjected to an experimental procedure and responsiveness again determined. Adrenaline and nor-adrenaline were used as test substances because the former has considerable cardiac action while the latter acts more peripherally. Nicotine probably acts chiefly on the nervous system, although it has many other effects. Barium chloride was used because it has its primary action on peripheral arteries and arterioles. Renin and angiotonin were of special interest to us because of their possible relationship to hypertension. Tetraethylammonium chloride (TEA) has multiple effects on the vascular tree. Injection of large amounts of TEA blocks transmission at autonomic ganglia, and augments the responsiveness of blood vessels to pressor drugs, including renin. It also causes liberation of a nor-adrenaline-like substance chiefly from the liver, which tends to counteract the fall in arterial pressure induced by autonomic blockade (1).

Our study of the problem of the factors which control the reactivity or responsiveness of heart and blood vessels as measured by pressor or depressor responses has

shown that the nervous system and the kidneys both may play an important part (2, 3). Since the classic work of Mann, Essex and Bollmann on the effects of operative removal of the liver, the function of this organ in protein and carbohydrate metabolism has been extensively studied. Though the manner of death of the animals has been carefully documented, no explanation of its mechanism is apparent. Our views on this question are based principally on indirect evidence.

In our search 10 years ago for the source of renin-substrate, attention was naturally directed chiefly to the liver. It was shown that hepatectomy reduced the substrate concentration of the plasma and that the response to renin disappeared. The loss of renin response could not have been due alone to inhibition of the response to angiotonin, the reaction product of the enzyme system renin—renin-substrate, because the latter was still present, though often diminished. Incidentally, we noticed that the response to adrenaline was greatly reduced but paid no further attention until work was begun on shock during the war. It was soon found that in shock, produced by a variety of means, one of the most characteristic changes was the appearance of vascular refractoriness (4).

In order to facilitate understanding of the experiments reported here, a brief sketch of their plan is given.

TABLE 1. RESPONSE OF NORMAL DOGS TO VASOACTIVE AGENTS

	MM. HG		MM. HG
Adrenaline.....	+45	TEA.....	+10-38
DL-nor-adrenaline.....	+80	Angiotonin.....	+17
Barium chloride.....	+22	Histamine.....	-42
Nicotine.....	+44	Renin.....	+40

The chief reason so many experiments were required is the normal variability of the response, as we have pointed out in detail elsewhere (5). The normal responses (table 1) were derived from studies, repeatedly made in many cases, on 65 dogs under sodium pentobarbital anesthesia. They furnish the basis for comparison with the responses after experimental procedures.

After hepatectomy, the responses to most substances are sharply reduced. The fact that renin responsiveness is especially quickly lost brings up the problem of the mechanism of tachyphylaxis to renin. The experiments in which the kidneys were removed before hepatectomy were designed to illuminate this problem and showed that nephrectomy prevented the initial loss of response to renin after hepatectomy. Many attempts were made to restore responsiveness after hepatectomy. The only partial success achieved (when refractoriness was established) was with TEA injections and cross-circulation of large amounts of blood from normal or nephrectomized dogs. Refractoriness elicited by hepatectomy could in part be prevented by first increasing reactivity by partial spinal cord destruction and after a few days of recovery, removal of the liver. Simple denervation of the liver by stripping the hepatic artery did not augment responsiveness, hence the effect of cord destruction is mediated by another mechanism. Thus the loss of response due to hepatectomy is demonstrated but the mechanism is not elucidated by these experiments.

METHODS

Details of most of the methods used have been published (5). The hepatectomies were performed by Dr. John Reinhard, Dr. Ralph Prince and Mr. William West, all of whom have had a large experience. Usually it has been found by those who have worked with hepatectomized dogs, experience in the operative technique and pre- and post-operative care are vital to success of the experiment. The results described are drawn from 110 successful hepatectomies.

The technique of hepatectomy was that of Firor and Stinson (6) with certain additions. After 24 hours starvation, atropine grains 1/100 were given. Under light ether anesthesia, 200 cc. of 10 per cent glucose was given at a rate of 8 cc/minute. After operation the blood sugar level was maintained by injecting 0.25 gm. of glucose/kg/hour. Heparinized blood (about 200 cc., the amount depending on the bleeding encountered) was given intravenously during operation or directly after it. An attempt was made to keep blood volume, sugar and sodium chloride normal after operation.

The dosages of the test drugs were 1) adrenaline 20 μ g.; 2) 0.15 cc. nicotine 1:1000 dilution; 3) DL-nor-adrenaline 25 μ g.; 4) 0.5 cc. barium chloride 18 mg/cc.; 5) tetraethylammonium chloride (TEA) 2.5 to 5 mg/kg.; 6) angiotonin 5 units; 7) 0.04 mg. histamine. Renin substrate was prepared by the method of Plentl and Page (7), renin by that of Helmer and Page (8) and angiotonin by Page and Helmer's method (9). Mean arterial pressure was measured by a mercury manometer connected to the femoral artery. Injections were made into the femoral vein.

RESULTS

Adrenaline, Nor-adrenaline, Barium Chloride, Nicotine, and Pituitrin. Anesthesia was rarely required during the tests of responsiveness. Some animals, after recovery from the ether used during hepatectomy, walked or frisked around the room, drank water and seemed comfortable. Most of them, however, were content to lie quietly while the femoral artery was being cannulated under procaine anesthesia. An hour or more before death, the animals became very quiet and arterial pressure began its fall. Convulsions usually did not occur unless the blood sugar had been allowed to decline to low levels. We have never been able to prolong the life of the animal to any significant degree by any of the many methods tried. Even repeated cross-circulation of blood with a healthy partner in the circuit was without avail.

The most striking change brought on by hepatectomy was the loss of reactivity of the vascular tree to a wide variety of drugs and an increased sensitivity to a few. Refractoriness appears within an hour or less after operation and usually lasts and may intensify until death.

Among the 110 animals studied it was unusual to observe responses of the order obtained in normal animals, though an occasional dog responded normally to some of the test substances. The responses to initial injections of adrenaline and nor-adrenaline were usually greatly reduced and often obliterated altogether. Expected responses in normal dogs of +45 and +80 mm. Hg for adrenaline and nor-adrenaline were reduced to +12 and +20 mm. Hg, respectively.

The response to nicotine was studied with the hope that some regular pattern might emerge in spite of its complex action. None did. Fall or rise in arterial pressure occurred with a preponderance on the side of fall. Biphasic curves were also often observed with the rise preceding the fall in most cases, but this was reversed in some. Perhaps the most significant thing about the action of nicotine was that, despite the refractoriness to other drugs, the response to this substance was usually sharp. For example, the response to adrenaline was 12 mm. Hg with 45 mm. Hg expected in normal animals, but with nicotine a rise of 58 mm. Hg occurred with an expected rise of 44 mm. Hg in normal animals.

TABLE 2. EXAMPLES OF EFFECT OF HEPATECTOMY ON VASCULAR REACTIVITY

EXPER. NO.	ADREN- ALINE	NICOTINE	HISTAMINE	BaCl ₂	ANGIO- TONIN	RENIN	TEA	B.P.	TIME AFTER OPERATION
	mm. Hg								hr. min.
262	36	78	-20	38	10	o	+8-20	124	3 10
	46			24			+14	118	
	44						+8	118	
	26							100	
	20	o		48	o			120	
256	12	58		16	14	o	-80	116	4 5
	20			32	6			40	
	38			22	12			66	
	24				16		-54	78	
	22	o				o		42	
	36							44	
253	8	+32-30	-48	14	8	o	-48	132	3 5
	10			24			o	44	
	12							64	
	18							78	
	24						+10	86	
	26				12		+16-110	128	
252	18	58		10		o	+10-14	76	4 35
	18							66	
	8								
247	6	+24-22		12	16	o	-30	88	5 30
	14							98	
	18							50	
				38				50	
	26			22	4	o	+8	76	
	26			-22				61	
225	10			4		o	-34	110	2 7
	6							60	
	4							42	
213	+4-6	+8-20		8	6	14	-58	110	1 35
	22				4			61	
	30				4			72	
	6				12			40	
207	4	18		12	o	o	-28	78	1 45
	32			32	10			74	
	46			40	16		+10	80	
208	+6-12	22	-12	14	6	o	+4-54	128	1 21
	4			o				104	
	o			6				84	
205	o	24	-4	22	6	o	-28	90	2
	4			o	18			94	
199	10	32	-14	10	o	o	-24	62	2 18
	4				o				
	8				o				

The response to histamine was reduced, -20 mm. Hg as compared to -42 mm. Hg expected in normal dogs. Barium chloride was similarly depressed, $+15$ mm. Hg compared with an expected $+22$ mm. Hg. These are average figures, the normal ones being derived from a study of 65 animals under sodium pentobarbital anesthesia (5). On a number of occasions after testing the dog without anesthesia, intravenous pentobarbital was administered and the tests repeated. With all the drugs used except nicotine, the responses were not greatly altered, provided no serious fall in arterial pressure occurred when they diminished. Regardless of pressure, the response to nicotine was usually significantly reduced by anesthesia.

Pituitrin was another drug which often gave active responses even though most of the other drugs exhibited diminished responses. Indeed, as is so common in studies on vascular reactivity, each drug may on occasion follow a pattern of its own. In this particular study, barium chloride, nicotine and pituitrin often caused sharp changes in arterial pressure when the other drugs were almost without action. But they could not be depended upon always to do so.

Renin and Angiotonin. Renin produced little or no response in 37 hepatectomized dogs but the angiotonin response was only moderately reduced and, in some, quite normal. Administration of tetraethylammonium chloride (TEA) did not restore the lost response to renin, nor to angiotonin, when it was impaired.

As a control experiment, the spleens of 7 dogs were removed, 2 feet of bowel resected and anastomosed end to end, followed by clamping of the inferior vena cava and portal vein for 5 minutes. The aim was to produce an amount of trauma somewhat comparable to that from hepatectomy. But an hour and a half after recovery from the operation, the renin response was excellent, indeed, much better proportionately than the adrenaline response.

Action of TEA. Since we had shown (10) that injection of large doses (10–20 mg/kg.) of TEA into normal dogs augments greatly the response to adrenaline, noradrenaline, barium chloride and other substances, it was of importance to determine whether or not it would similarly affect the responses in the more or less refractory hepatectomized animals.

It was immediately apparent that the initial falls in blood pressure produced by TEA were usually much greater than normal and, further, recovery to control levels was unusually slow. The results of many injections show conclusively that most hepatectomized animals are abnormally sensitive to the depressor action of TEA

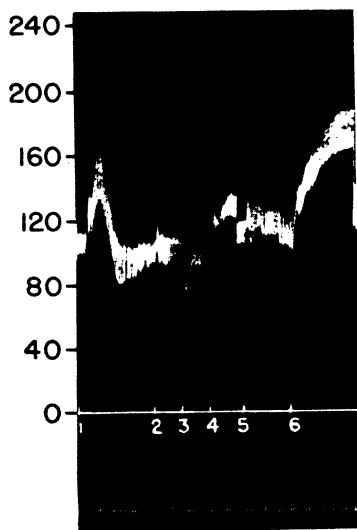


Fig. 1. EXAMPLE OF RESPONSES of normal dog to 1) adrenaline; 2) nicotine; 3) histamine; 4) barium chloride; 5) angiotonin; 6) renin. The same doses were used as in the experiments on hepatectomized dogs. (See fig. 2.)

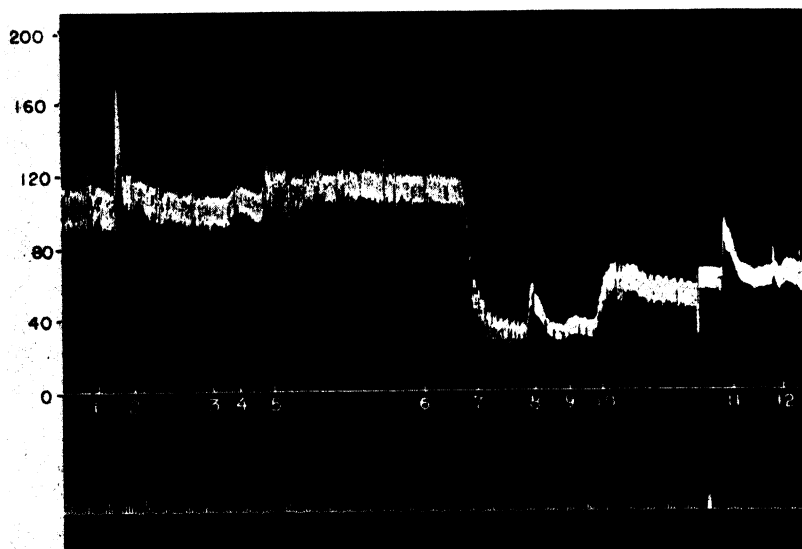


Fig. 2. EXAMPLE OF VASCULAR REFRACTORINESS in hepatectomized dog 256. Hepatectomy completed 11:10 A.M., experiment conducted 3:15 P.M. in an 18-kg. dog. Received 2000 cc. 5% glucose in saline, 750 cc. saline and 150 cc. blood. 1) Adrenaline; 2) nicotine; 3) histamine; 4) angiotonin; 5) barium chloride; 6) renin; 7) TEA 5 mg/kg.; 8) adrenaline; 9) angiotonin; 10) barium chloride; 11) adrenaline; 12) nicotine.

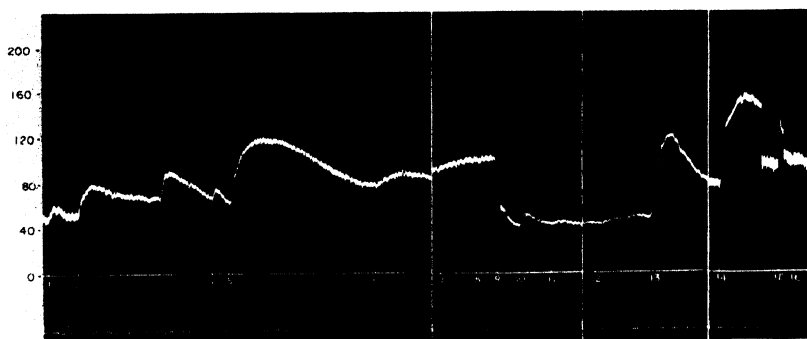


Fig. 3. EXAMPLE OF NEPHRECTOMY followed by hepatectomy on vascular responsiveness (dog 249). Nephrectomy completed 9 A.M., hepatectomy 10 A.M., experiment conducted 2:30 P.M. in a 11.7-kg. dog. Hb 91% of normal one half hour after hepatectomy and 104% at 3:15 P.M. Total blood given 115 cc., 1000 cc. 5% glucose in saline. 1) Adrenaline; 2) barium chloride; 3) angiotonin; 4) adrenaline, infusion of blood started; 5, 6, 7) renin; 8) infusion of blood stopped; 9) TEA 50 mg.; 10) adrenaline; 11) angiotonine; 12) renin; 13) barium chloride; 14) obstetrical pituitrin $2\frac{1}{2}$ units; 15) adrenaline; 16) renin.

Even after repeated injections there was only a small tendency to reverse from depressor to pressor action as occurs in normal dogs.

Significant augmentation of pressor responsiveness was rarely observed. In an occasional experiment, however, it occurred in some measure immediately after

injection of the TEA. More usual was a gradual increase in responsiveness over several hours, during which repeated injections of TEA were being made. But in the majority of experiments especially where hepatectomy alone was involved, TEA was ineffective in either aiding in the appearance of responsiveness when it was not there or in increasing it when it was. This is the second example we have seen where TEA was ineffective in augmenting the activity of vasoactive substances. The first was during deep shock which, in our experience, is always coupled with refractoriness.

Adrenaline and nor-adrenaline were among the drugs most readily augmented. Renin, on the other hand, was among the most difficult. In a few experiments after several hours of repeated injections of TEA, better responses to renin occurred. But they were rare and might have been due to other factors, so that we are not sure that TEA was responsible. Certainly once responsiveness to renin is lost, the injection of TEA will rarely restore it.

Combinations of Nephrectomy, Hepatectomy and Adrenalectomy. Removal of both kidneys just before the hepatectomy greatly changed the responsiveness of hepatectomized dogs, especially to renin. A good response to renin occurred on its first injection. When nephrectomy was performed 2 days before hepatectomy, responsiveness to renin usually was enhanced well above that when nephrectomy directly preceded hepatectomy. This is in line with the late augmenting effect of nephrectomy. A second dose gave about half the initial response and the third little or none. Renin responsiveness was not restored except occasionally by cross-circulation of fresh blood from normal or nephrectomized dogs alone or supplemented by TEA. TEA frequently augmented to a moderate degree responses to adrenaline, nor-adrenaline and barium chloride, but was largely ineffective with renin and angiotonin.

A few experiments were done in which the order of the operations was reversed, namely hepatectomy followed immediately by nephrectomy. The results differed only in that renin responses did not seem quite as active as after prior nephrectomy. The differences appeared to be quantitative rather than qualitative.

Three experiments were performed in which adrenalectomy and nephrectomy preceded hepatectomy. The responses to adrenaline and nor-adrenaline were what would be expected in a nephrectomized-hepatectomized animal. Cross-circulation of 800 cc. of heparinized blood with a nephrectomized dog, the cord of which had been destroyed from C₆ caudad, did not increase responsiveness despite the fact that the responses in the donor were about 4 times as great as in the hepatectomized recipient.

The effects of precedent nephrectomy on the actions of other drugs were not as striking as with renin. In spite of good renin responses, adrenaline responses were only slightly increased. Barium chloride and angiotonin seemed somewhat improved as compared with their actions in animals hepatectomized alone. Surely the most impressive effect of nephrectomy preceding hepatectomy is the restoration, if only for a few injections, of a renin responsiveness which is lost when hepatectomy is performed with the kidneys intact. Hepatectomy is thus shown to accelerate renin tachyphylaxis.

Spinal Cord Destruction, Hepatectomy, Nephrectomy. Section of the spinal cord at C₆ and destruction caudad when done under sterile conditions and when recovery

from spinal shock is completed, greatly increases responsiveness (2). The effects of hepatectomy are of interest in such preparations.

The cord was destroyed 3 days before hepatectomy. Since the animals were paralyzed, a general anesthetic was not required. An example of such an experiment is given in table 4. The results suggest an average between the heightened sensitivity caused by cord destruction and the vascular sluggishness which results from hepatectomy. Thus adrenaline, barium chloride and angiotonin all showed about normal but not heightened response. TEA, which yielded a pure pressor response in the cord-destroyed dog and a pure fall in the hepatectomized animal, now elicited little or no change in arterial pressure. The inhibiting action of hepatectomy on renin prevailed and despite good responses from angiotonin, renin was without effect.

When nephrectomy as well as cord destruction preceded hepatectomy, the results were again what might have been anticipated. The responses to adrenaline, barium chloride, angiotonin, etc., were about the same or slightly increased as without nephrectomy but small renin responses were now obtained. When the renin response was exhausted as the result of repeating the injections, 0.4 gm. of purified renin-substrate solution was infused but it proved to be without effect in restoring the renin response. TEA in small doses produced a severe fall in blood pressure with some augmentation after repeated doses had been given. Renin elicited a sharp rise in pressure but on repetition, the response disappeared. Pithing did not further increase the responses to adrenaline or nor-adrenaline. Tetramethyl ammonium chloride, as distinguished from TEA (10 mg.) was strongly pressor as was obstetrical pituitrin (2 U.). Towards the end of a long experiment when refractoriness was appearing, prostigmine (0.25 mg.), ouabain, sodium bicarbonate and magnesium chloride were without effect on responsiveness.

Removal of the adrenal glands showed that under the circumstances of these particular experiments they did not appear to have any decisive part in the intensity of the vascular responses elicited.

Effect of Cross-Circulation. A series of 11 experiments was performed in which an effort was made in nephrectomized-hepatectomized dogs to restore the responsiveness especially of renin and adrenaline, by means of cross-circulation with normal or nephrectomized dogs. The technique has previously been described (11). From one to 6 l. of blood was crossed. In 6 of the experiments no increase occurred. There was a slight increase in response to renin in one and significant increases in 2 others. The responses to adrenaline were definitely increased in most cases.

When TEA was also injected, augmentation of the adrenaline responses occurred in some but not in other animals. It was, however, seldom impressive. We were unable to distinguish from the height of the arterial pressure between those that would be augmented and those that would not. If it were below 50 mm. Hg it seldom occurred.

When nephrectomy did not precede hepatectomy, neither cross-circulation nor TEA had any significant effect on the renin response. Clearly, neither cross-circulation, which must have supplied renin-substrate in abundance, nor TEA were able regularly to restore responsiveness to renin nor to augment adrenaline. But that they can occasionally help is shown by an experiment illustrated in table 5. The effect is usually not nearly as great as in this example.

TABLE 3. EXAMPLES OF EFFECT OF NEPHRECTOMY AND HEPATECTOMY ON VASCULAR REACTIVITY

EXPER. NO.	ADRENALINE	NICOTINE	HISTAMINE	BaCl ₂	ANGIOTONIN	RENIN	TEA	B.P.	TIME AFTER NEPHRECTOMY	TIME AFTER HEPATECTOMY
	mm. Hg								hr. min.	hr. min.
219	14				24	48		94	2 53	1 40
	+10-14				10	22	-82	126		
	36				4	0		60		
	46			52				68		
	38				8	8		100		
	32				0	0	-20	64		
	34			30		0		56		
	14							76		
	44				0	0	0	84		
	12							68		
222	10	-26-+30	-44	30		56		130	4 0	3 30
	0					26	-80	114		
	8			24				38		
	18				0	0		60		
	8							54		
	34				12	0		70		
	30			32	14			80		
249	8			26	24	58	-62	70	1 0	4 30
	10	8		74	0	6		50		
	36			38		0		80		
	18							54		
257	20	48	-24		34	58		88	4 30	3 45
	10			32	0		-78	126		
	20			60	12			52		
	12					14		38		
259	10	38	-16	20	16	22	-66	88	5 45	5 0
	8			40	6	8	-10	56		
	14					12	-6	76		
	6	0		0		20		80		
269	16	30	-14	20	32	28	-44	140	3 50	3 10
	40			46	0	0	-12	120		
	14			28	6	0	0	84		
	8									
345	16					58	-46	102	3 10	2 25
	46						14	82		
	54						22	88		
	58					20		88		
349	22				16		-50	124	4	3 30
	50				14	26		100		

If post-hepatectomy refractoriness to renin is due in part only to substrate lack or to inhibition by the nervous system, it may be asked whether or not it is due to lack of response to angiotonin. The results of repeated angiotonin injection into 58 hepatectomized or nephrectomized-hepatectomized dogs show little direct parallelism between response to angiotonin and to renin. We have never seen a response to renin when there was none to angiotonin but the reverse is often true. Angiotonin responsiveness seems more to resemble adrenaline responsiveness but there is no direct parallelism. Adrenaline response often occurs without a response to angiotonin, but the reverse is not common.

Other Factors Associated with Vascular Responsiveness After Hepatectomy.

1. *Height of the arterial pressure.* No direct relationship was evident between height of arterial pressure and the responsiveness after hepatectomy or nephrectomy-hepatectomy. There was a tendency for the animals with pressures lower than 65 mm. Hg to show almost no response to a variety of vasoactive drugs but low pressures do not necessitate refractoriness. Perhaps the most convincing observation was that persistence of arterial pressure at 100 mm. Hg or above gave no assurance against almost complete refractoriness. It seems safe to conclude that while the height of the arterial pressure has a modifying influence, especially when persistently low, it is not decisive after hepatectomy.

In order to test this belief further, the blood pressure of a hepatectomized dog was raised by an infusion of large amounts of nor-adrenaline from 42 mm. Hg to 106 mm. Hg and kept at that level for 30 minutes. Refractoriness was not overcome and no significant change in the responses to adrenaline, TEA or barium chloride noted.

One of the striking effects of persistently lowered arterial pressure is the damping influence it has on the depressor action of TEA. The proportionate fall may however be as great as when the pressure is normal or elevated.

2. *Struggling and asphyxia.* In normal dogs, struggling may be associated with a rise or fall in arterial pressure or a combined effect. In our unanesthetized, hepatectomized dogs, it was seldom associated with either to any significant extent. It is as though the nervous system no longer was able to respond or else the stimulus was not produced. The response to asphyxia was also greatly damped.

3. *Other factors without decisive influence on reactivity.* Hematocrit-reading and plasma-protein (electrophoresis method) determinations have been made throughout a number of the experiments but revealed no correlation with vascular responsiveness (12). Administration of glucose, saline, sodium bicarbonate, magnesium chloride, calcium gluconate, 6 percent protein hydrolysate infusion, gelatin infusion and arterial transfusion were without decisive influence. The intravenous injection of calcium pantothenate (100 mg.), riboflavin (500 mg.), d-isoascorbic acid (100 mg.) pyridoxin hydrochloride (50 mg.), ascorbic acid (500 mg.), nicotinic acid (100 mg.), sodium p-aminobenzoate (5 cc. 10 percent solution) and sodium beta-glycerophosphate (100 mg.) were also without effect.

DISCUSSION

That the liver might have a part in the control of the reactivity of the blood vessels and heart seems not to have been recognized. The experiments we have de-

scribed clearly indicate that for many vasoactive substances, vascular reactivity falls to a low level whether the substance be pressor or depressor.

A certain degree of specificity was observed during refractory states, some drugs giving little or no response while a few others such as TEA exhibited an increase. After hepatectomy the drugs which tended to preserve their activity to a greater or lesser degree were barium chloride, nicotine, angiotonin and pituitrin. In contrast, renin lost its activity unusually easily. These facts suggest that the refractory state is concerned with specific chemical actions in vascular muscle.

Posthepatectomy irresponsiveness is a state not easy to overcome. In cross-circulation experiments amounts of blood crossed up to 6 l. from normal or nephrectomized dogs seemed to aid in restoring reactivity. But there were more failures than

TABLE 4. EXAMPLE OF EFFECT OF HEPATECTOMY 3 DAYS AFTER SPINAL CORD DESTRUCTION (C₆)¹

DRUG	TIME	B.P.	RES- PONSE	DRUG	TIME	B.P.	RES- PONSE
		mm. Hg	mm. Hg			mm. Hg	mm. Hg
Adrenaline	9:05	106	20	Angiotonin	10:48	114	12
Barium chloride	9:12	108	14	Renin	10:56	112	0
Angiotonin	9:16	108	12	TEA 5 mg/kg.	11:14	82	0
Renin	9:21	100	4	Adrenaline	11:17	92	42
<i>Carotid sinus inactivation and vagotomy</i>				Barium chloride	11:27	104	12
Adrenaline	9:46	92	34	<i>300 cc. fresh heparinized normal dog's blood</i>			
Barium chloride	9:51	100	10	Renin	1:00	102	0
Angiotonin	9:55	100	12	Adrenaline	1:03	112	24
Renin	10:00	100	0	Angiotonin	1:06	114	20
TEA 5 mg/kg.	10:19	82	0	Renin	1:08	102	0
TEA 5 mg/kg.	10:27	74	10	Adrenaline	2:32	70	32
Barium chloride	10:35	84	18	Barium chloride	2:35	74	0
Adrenaline	10:43	102	48				

¹ Dog 756. Hepatectomy completed 7:30 A.M.

successes. Thus, it is improbable that there is a deficiency of a hypothetical substance contained in small amounts in normal blood which overcomes it. The most that can be said is that cross-circulation with animals in the circuit with intact livers sooner or later appears to aid in restoring vascular reactivity.

We have found no way to prolong the lives of these animals. Twelve to 16 hours after operation, the animals become abnormally quiet, vascular reactivity further diminishes, blood pressure falls and death ensues in an hour or more. This premortal period is in sharp contrast with that several hours after operation when the dogs appear quite normal.

The refractoriness after hepatectomy is similar to that described during shock (13) and, in view of the many defects of hepato-portal circulation and function in shock, may well be of similar nature. Just as in shock, arterial pressure need not fall

until well after the refractory state has developed. Vascular collapse can and does occur regularly after an hour or more of relatively complete loss of reactivity.

The response to certain vasoactive substances requires comment. The response to both adrenaline and nor-adrenaline quickly diminished after hepatectomy and was most readily restored by cross-circulation and TEA. As in shock, these substances form excellent indicators of the reactive state of the vascular tree.

It is in some ways surprising that removal of the liver reduces rather than elevates vascular reactivity. The liver has been considered an organ adapted to detoxication. Concerned with the removal of substances from the blood, it could aid in conjugating or destroying of substances such as adrenaline and nor-adrenaline. If they were not removed by the liver, the response after hepatectomy should be greater, not less.

The responses to nicotine were irregular, and seemed more indicative of changes in the nervous system than any other single bodily system. The most striking observation was that when animals were almost refractory to adrenaline, nor-adrenaline, etc., they often responded to nicotine. Barium chloride also exhibited wide and often unpredictable responses. Its action was often augmented by TEA out of proportion to that of other substances. Pituitrin also was a substance which gave responses in otherwise almost refractory animals. But this was not always true. We have been constantly impressed by the high degree of specificity in its response to chemical stimulation exhibited by vascular muscle.

Of especial interest to us is the response to renin and angiotonin. Ten years ago we (14) searched for the source of renin-substrate and quite naturally looked to the liver. Substrate concentration was shown to be diminished by hepatectomy. But the operation does not, correspondingly, inhibit the response to angiotonin, a fact confirmed in our present data. Leloir and co-workers (15) found further that abdominal evisceration did not reduce plasma renin-substrate concentration. But renin injection into eviscerated dogs was followed by its permanent disappearance. The same procedure in nephrectomized dogs caused only a transitory decrease. Thus it seems that during the operation of hepatectomy, the kidneys secrete enough renin to make the animal's own vascular tree refractory to it.

Results presented in this paper extend these earlier observations. Renin rarely produces any response after hepatectomy, nor is responsiveness significantly restored by cross-circulation, thus supplying ample substrate. In occasional animals, TEA has appeared to restore in part some responsiveness, but this is unusual.

In contrast to these results, when the kidneys were removed directly before the hepatectomy was performed, response to renin was almost normal. The initial response was good, but on repetition it quickly faded out. The pressor action of angiotonin usually remained even after loss of the renin response. In contrast with hepatectomized animals, cross-circulation and TEA were much more likely to augment responsiveness.

It seems to us that the most reasonable explanation is that during the operation renin is discharged from the kidneys in increased amounts, eliciting tachyphylaxis. Since the source of renin-substrate has been removed in removing the liver, the blood concentration falls. Now the injection of renin exhibits no effect for at least 2 reasons:

1) the vessels are already tachyphylactic and 2) the concentration of substrate is low and quickly used up by the injected renin. Removal of the kidneys before hepatectomy probably has a multiple effect: 1) removal of the source of renin which produces tachyphylaxis; 2) sensitization to renin which occurs quite independently of hepatectomy; 3) removal of an organ which can either destroy, excrete or inhibit renin. Of these three, we are of the opinion that the first is the most important, in spite of the fact that in control experiments aimed to produce amounts of trauma comparable to hepatectomy, responsiveness to renin persisted. The reason cross-circulation of blood or injection of blood or purified renin-substrate does not restore renin response is that the vessels are already tachyphylactic. Even in normal animals we have not been able to overcome tachyphylaxis by blood transfusion or injection of purified

TABLE 5. EXAMPLE OF EFFECT OF CROSS-CIRCULATION OF HEPATECTOMIZED AND NORMAL DOG ON VASCULAR REACTIVITY OF THE HEPATECTOMIZED ANIMAL¹

TIME	ADREN- ALINE	RENIN	TEA	B.P.	VOL- UME OF BLOOD CROSSED	TIME	ADREN- ALINE	RENIN	TEA	B.P.	VOL- UME OF BLOOD CROSSED
			5 mg/ kg.		cc.				5 mg/ kg.		cc.
2:05	10			102	0	3:39		0		66	2800
2:07		18		108	0	3:41	62			66	3000
2:20		6		106	0	4:25			8	76	3450
2:22	Continuous cross-circulation begun					4:26	Cross-circulation stopped				
2:37	20			100	600	4:28		20		74	
2:41	28			92	720	4:35	32			88	
2:45		6		90	900	4:40	28			80	
2:50			-30	92	1050	4:44			0	80	
2:53	40			66	1170	4:47		12		78	
2:58		0	0	72	1500	4:58		22		82	
3:04				80	1740	5:08	48			110 ²	
3:07	46			78	2000						
3:37			0	76	2640						

¹ Dog 365. Hepatectomy completed 10:30 A.M.; dog wgt. 8.3 kg.

² Artificial respiration was begun.

substrate. At most, transfusion and/or TEA injections merely aid in restoring the renin response but are not decisive.

The problem of renin tachyphylaxis must also be touched on because of the surprisingly specific effect hepatectomy has on the response to renin. A variety of attempts have been made to restore the renin response, the most significant of which was cross-circulation of from 4 to 6 l. of blood with normal or nephrectomized dogs or dogs functionally denervated by large doses of TEA.

Since renin-substrate concentration in the plasma appears to fall after hepatectomy, cross-circulation should have restored ample amounts for the renin response. But the renin response did not reappear. Nor did intravenous administration of purified renin-substrate aid it (2). Since the pressor action of angiotonin is not necessarily lost when renin elicits no response, we must conclude that neither lack of

substrate nor lack of angiotonin responsiveness is wholly responsible for the phenomenon of tachyphylaxis. The problem currently seems to center on the nature of the changes in the arterial musculature itself.

The impression should not be conveyed that cross-circulation had no effect on responsiveness. It increased it in some cases, but not regularly nor greatly enough to consider fresh blood a decisive factor in overcoming tachyphylaxis.

TEA also aids in an irregular and moderate degree in overcoming tachyphylaxis; therefore, autonomic blockade is rarely a major part of the renin tachyphylaxis of hepatectomized dogs. With such substances as adrenaline, nor-adrenaline and barium chloride, where no tachyphylaxis is involved, both cross-circulation and TEA were likely to increase their responsiveness, but never to the degree seen in normal or nephrectomized animals.

Spinal cord destruction from C₆ caudad, when the animal is allowed to recover fully for a day or two, is followed by greatly heightened responsiveness including that to renin. This is in all probability due to loss of the inhibitory action of the nervous system on the vascular system, such as that exhibited by the carotid sinus mechanism. When hepatectomy was performed on such animals, the response to renin disappeared and that to adrenaline, nor-adrenaline and barium chloride was greatly reduced. Transfusion of fresh blood and TEA did not restore the renin response and only slightly increased that of the other drug. Addition of nephrectomy preceding hepatectomy did what might have been anticipated, namely, slightly increased the adrenaline, nor-adrenaline response and caused reappearance of small renin responses. Thus it is suggested that the heightening effect of the loss of inhibitory action of the nervous system tends to be counteracted by the refractoriness associated with the loss of hepatic function, and even though the mechanisms may be different, the response is simply the resultant of the two opposing forces.

The adrenal glands under the circumstances of our experiments did not play a decisive role.

There remains to be discussed the problem of why hepatectomy produces refractoriness of a type which can be modified but not fundamentally altered by destruction of the nervous system, autonomic blockade and nephrectomy. The height of the arterial pressure does not play a decisive part, nor do blood volume, hematocrit, CO₂-combining power, sugar and amino acid content of the blood. In short, nothing we have found except the kidneys and nervous system exerts much influence on this special type of refractoriness. There is no evidence on which to decide whether or not the vascular tree lacks something important for its metabolism obtainable from the liver or whether because the liver is not doing its job, toxic substances appear in the blood to injure blood vessels.

Refractoriness seems always to appear before death following hepatectomy and probably is an important mechanism leading to it. We believe the same is true of oligemic and other types of shock. But it would be wrong to suppose that partial loss of vascular responsiveness, as we measure it, is the only obligate path along which dissolution proceeds. For many hours after hepatectomy, the vascular tree, despite relative loss of sensitivity to chemical stimuli, is able to adjust normally to

prolonged periods of severe hypotension and recover following reinfusion of the withdrawn blood (16). Shocked animals may survive long after development of moderate refractoriness which does not, as might be supposed, herald immediate death. But over long periods it is doubtful that a refractory vascular tree could do its job. The ability of the blood vessels to contribute their share in the struggle for existence is lost when the liver is removed.

SUMMARY

Hepatectomy in dogs greatly reduces the responses to adrenaline, nor-adrenaline, barium chloride, histamine and renin, less so to nicotine, angiotonin and pituitrin, and actually increases the depressor action of TEA. This refractoriness is not overcome by cross-circulation of large amounts of blood from normal animals nor by injection of large doses of TEA.

Hepatectomy immediately preceded by nephrectomy reduces the responses less, especially those to renin. Initially, renin may give a full response but on repeated injection it fades quickly. Cross-circulation and TEA injection seem to aid in some degree restoration of partial responsiveness. Adrenalectomy under the conditions of these experiments does not have a significant action. Spinal cord destruction, which augments pressor responses, tends to counteract the depressing effects of hepatectomy, the response being the resultant of the 2 opposing forces. Nephrectomy a day or more preceding hepatectomy augments the force favoring pressor response. The height of the arterial pressure and injection of a variety of substances which might be supposed to be concerned in the metabolism of the muscle of the blood vessels were without influence on response in hepatectomized animals. Renin tachyphylaxis in hepatectomized or nephrectomized-hepatectomized animals is not wholly overcome by injection of partially purified renin substrate or whole blood, nor by blockade of the autonomic nervous system by TEA. Tachyphylaxis occurs even when the response to angiotonin is adequate.

CONCLUSIONS

The liver participates in the mechanism concerned with the control of the responsiveness of the cardiovascular system to vasoactive substances. Loss of its influence leads to a refractory state, overcome only to a small degree by fresh blood and TEA. The nervous system, on the contrary, tends to reduce responsiveness because when destroyed, pressor-depressor action is greatly augmented. When hepatectomy and spinal cord destruction are combined the net effect despite the different mechanisms concerned seems to be a resultant of the opposing forces. The kidneys also act to reduce responsiveness, for on their removal, it increases. Tachyphylaxis to renin is caused by multiple factors, among them being reduction of renin-substrate, inhibition by the autonomic nervous system, and kidneys. The lack of response after hepatectomy seems chiefly caused by tachyphylaxis, resulting from renin excreted during and after the operation of hepatectomy along with exhaustion of renin-substrate by repeated renin injections.

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RELATIONSHIP OF PORTAL HYPERTENSION AND IRREVERSIBILITY OF SHOCK

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IN AN ingenious and complicated cross-circulation experiment, Frank, Seligman and Fine (1) were able to show that if the portal circulation were maintained at normal pressures in a dog, the systemic blood pressure of which had been



Fig. 1. SITE OF GRAFT showing method of handling of vein while being sutured to aorta

kept at shock levels, most of the animals recovered, while simply maintaining the same circulating volume by replacing the blood in the jugular vein rather than the portal vein resulted in a fatality in 15 of 17 controls. Since it is conceivable that the donor dog contributed some factor to the circulation of the shocked dog in these ex-

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periments, the experiments herewith reported were devised to obviate this difficulty and to test the theory of Frank *et al.*

Under morphine and sodium pentobarbital anesthesia, a long strip of the right jugular vein was removed, and the wound closed. Next with the animal lying on its left side, a long subcostal incision was made opening into the peritoneum and exposing the portal vein (fig. 1). The posterior peritoneum was freed, the vena cava retracted and the aorta exposed below the renals for a distance of 3 cm. Any branches



Fig. 2. GRAFT in place

at this level of the aorta and vena cava were cut and tied. The jugular vein was then threaded on the plunger of a tuberculin luer syringe, being careful that any valves present in the vein would not be directed against the flow of blood from aorta. The aorta was then clamped above and below the freed area and a 4-mm. incision made into it. The jugular vein was then anastomosed to the aorta in the classical manner. The vein was then brought through the peritoneum to the portal vein. Either a large branch of the portal vein or the main vein was sutured to the jugular vein.

It is immediately apparent if the graft will function satisfactorily as the arterial

blood can be seen to distend the graft at once and that the red blood is mixing with the darker portal blood in the portal vein.

The carotid artery was then connected with a valve which would keep the arterial pressure at any given level as described by Kohlstaedt and Page (2). Because this operative procedure was quite long, a pressure of 40 to 50 mm. of Hg was used instead of 30 mm. Hg as used by Frank *et al.* The design of the Kohlstaedt-Page apparatus is such that the pressure readings are taken from a mercury U-tube manometer. The level oscillates with the heart beat. There is a definite lag in the oscillations so that absolute accuracy is not obtainable. The animals were kept at this shock level (40-50 mm. Hg) for an average of 2 hours, following which the blood remaining in the

TABLE 1

EXPERIMENT	PORTAL VENOUS PRESSURE IN CM. OF WATER			
	Normal	Fistula open 1 hour	Fistula open 2 weeks	Portal venous pressure ¹
1	9	42	39	22
2	8	41	34	26
3	8	36	30	21
4	10	31		23
5	7	34		20

¹ Before replacement of blood and during period of peripheral hypotension.

TABLE 2

NO. OF DOGS	SURVIVALS WITH FISTULAE	SURVIVALS CONTROLS
8	7	
17 ¹ (systolic BP 30 mm. Hg)		2
6 ¹ (systolic BP 40-50 mm. Hg, operative failure of fistula to function)		1

¹ Systolic pressure measured in mm. Hg by Kohlstaedt-Page valve.

bottle was pumped back into the animal, observing the precautions emphasized by Kohlstaedt and Page for intra-arterial transfusions.

All the venous-pressure measurements were made with an ordinary manometer using citrate solution and an 18-gauge needle for the vein. Readings were checked first from a level higher than the suspected pressure and then from a level lower than the determined pressure.

It is apparent from the results shown in table 1 that when the fistula is successfully constructed, there is an immediate rise in the portal venous pressure. In only 3 animals was the fistula open as proven by inspection at the end of 2 weeks' time. In all these 3, it was apparent that the lumen on the venous side of the anastomosis was constricting. However, the pressure in the portal vein was still high. In the acute experiments when the animals with satisfactory fistulae were put into shock by

bleeding from the carotid artery and the systolic blood pressure stabilized around 50 mm. Hg, the pressure in the portal vein still remained higher than the normal venous pressure as measured in the dog before establishment of the fistula, as shown in table 1, column 2. Thus the liver circulation was always assured a normal pressure.

Table 2 shows that under these circumstances, shock appeared to be reversible. Under the control experience of Frank *et al.*, and in our own experience where the fistula was technically a failure, 84 per cent of the animals (6 out of 7) did not recover after the acute hemorrhage.

As in the vivi-perfusion experiments a protective effect during hemorrhagic shock has been demonstrated which confirms the contention of Frank *et al.* that this protective effect is due to an improved blood flow through the liver. We have made no attempt to study the mechanism whereby this protective action takes place. The increased blood levels of catabolic products of the liver found in shock have been shown in clinical conditions in the absence of shock and hence are not specific.

SUMMARY

A method has been described whereby the portal venous pressure can be maintained at high levels. Under such circumstances in the acute experiment, shock, which in control animals is irreversible, becomes reversible.

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FUNCTIONAL SIGNIFICANCE OF RESIDUAL SYMPATHETIC PATHWAYS FOLLOWING VERIFIED LUMBAR SYMPATHECTOMY¹

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RELAPSE of patients following sympathetic denervation has been variously attributed to nerve regeneration, sensitization of blood vessels to humoral agents, development of intrinsic tone in vessels and incomplete denervation. Of these possibilities, the last is the subject of the study reported herein. The data suggest a re-evaluation of the significance of sensitization, regeneration and inherent tone in the effects of sympathectomy.

According to Smithwick (1) removal of sympathetic ganglia L₁ through L₃ results in denervation of the lower extremity, but Ulmer and Mayfield (2) suggest that the denervation may not be complete unless T₁₁ and T₁₂ are also removed. Ray and Console (3) report that even after total paravertebral sympathectomy, residual sympathetic activity persists, and this activity is abolished only after the appropriate anterior spinal roots are divided. We can confirm both in man and dog the observations of Cowley and Yeager (4) that the marked variability in anatomy of the lumbar sympathetic trunk creates doubt as to precise enumeration of ganglia removed at operation. Therefore some of the inconsistent results reported after sympathectomy may be due in part to anatomic variations.

Clinical observations such as are illustrated in figure 1 demonstrate the problem frequently presented following lumbar sympathectomy. In this hypertensive patient

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there was some immediate relief following operation, but at the time of the present observations the brachial blood pressure was still 210/126. Vasoconstriction in the toes occurred spontaneously (fig. 1) or could be induced by appropriate constrictor stimulation. At some time after sympathectomy, the feet became cold and pale and the patient complained of muscle cramping pains in the leg at rest. There was no claudication however. These pains were relieved by priscol, and carefully controlled observations revealed marked relaxation of vascular tone (table 1).

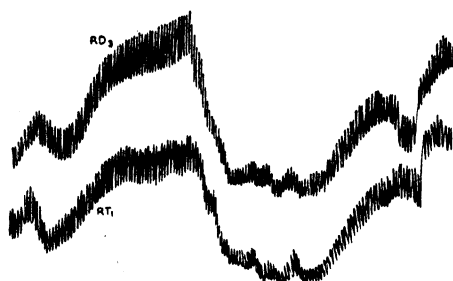


Fig. 1. PHOTOELECTRIC PLETHYSMOGRAMS illustrating spontaneous vascular dilations and constrictions in the normally innervated finger pad (top record designated as RD₂) and in the toe pad (lower record designated as RT₁) five years following bilateral lumbar sympathectomy.

TABLE 1. VASCULAR AND TEMPERATURE RESPONSES FOLLOWING INTRAMUSCULAR ADMINISTRATION OF PRISCOL IN THE SAME PATIENT REPRESENTED IN FIGURE 1

TIME	BLOOD FLOW IN FILTER UNITS		SKIN TEMPERATURE °C.		ROOM TEMPERATURE °C.	TIME	BLOOD FLOW IN FILTER UNITS		SKIN TEMPERATURE °C.		ROOM TEMPERATURE °C.
	RT ₁	RD ₂	RT ₂	RD ₄			RT ₁	RD ₂	RT ₂	RD ₄	
9:50	0.27	0.68	21.7	25.3	20.8	11:07	15 mg. priscol injected intramuscularly				
9:55	0.26	0.49	21.7	24.9	20.8						
10:06	0.44	0.61	21.5	23.7		11:11	0.67	2.70	22.0	28.5	19.5
10:14	0.29	0.59	21.1	23.2		11:29	0.95	2.64	23.2	30.5	
10:15	8 mg. priscol injected intramuscularly					11:35	1.13	2.74	24.0	30.8	20.0
10:17	0.54	1.66	21.1	23.3		11:56	0.93	1.92	25.8	30.4	
10:23	0.39	2.45	21.3	24.8		12:23	1.97	2.89	30.0	31.5	20.0
10:28	0.41	2.03	21.3	26.3	20.2	12:56	2.26	3.97	32.9	31.5	
10:29	16 mg. priscol injected intramuscularly					1:15	2.60	4.0	33.2	32.1	19.5
10:33	0.48	2.68	21.5	27.6		1:56	2.92	4.3	34.7	32.8	
10:40	0.43	1.96	21.9	29.0	20.1	2:30	3.00	4.98	34.0	33.8	
10:52	0.42	2.37	22.1	28.7		3:35	3.34	4.53	33.3	33.0	1.99
11:03	0.63	3.56	22.1	29.0							

As early as 1929 Richter (5) found it difficult to explain the gradual return of skin resistance values from immediately high values to lower levels following sympathectomy, while after section of all the peripheral nerves to the feet (monkey and cat) the effect was permanent. Roth and Craig (6) following careful analysis of several hundreds of sympathectomies at the Mayo Clinic confirm the report of Ray and Console (3). Anatomical studies have revealed intermediate ganglia lying within the gray and white communicating rami and their sites of origin on the spinal nerve and in the ventral primary ramus of the spinal nerve in the fetus and in adult man

(7-10). Fibers have been traced distalwards from these cells beyond the origin of the communications between spinal nerves and the paravertebral ganglia (10). In this manner, functionally significant conduction pathways exist which do not traverse the sympathetic chain, and which are not interrupted upon sympathetic trunk extirpation.

Selection of the dog for the initial studies on the functional significance of these accessory pathways was conditioned by the necessity of making observations without the complication of anesthesia, the necessity of checking functional recovery against anatomy at any required time, and by the ease with which carefully selected dogs may be trained.

METHODS

Vasomotor and sudomotor activity were studied on the large central pad of the hind paw by measuring skin temperatures by thermocouple, by recording vascular responses with the photoelectric plethysmograph (11) and sweating by means of the iodine-starch-paper technique (12). Blood flow levels were inferred from the correlation between the amplitude of the pad pulses and flow (13), but since the flow equivalent of the skin pulse of the dog has not been determined by calibration experiments, the amplitude of the pad pulse is recorded below in 'filter units,' the significance of which has been described in an earlier paper (13).

Observations were made at frequent intervals; 1) during a suitable preoperative period of training (one to 6 weeks), 2) while the animal was still under the anesthesia following operation, 3) several times daily for the first few days after operation and 4) daily thereafter for several weeks or months.

At the time of operation care was taken to verify the levels from which the sympathetic trunk was removed. Histological preparations were made of all excised tissue using both silver and hematoxylin-eosine techniques. Completeness of removal of the chain has been established in those animals which have thus far come to autopsy and complete serial section has been made of the spinal nerves with their communications. By similar histological study, the entire lumbar outflow in the dog has been carefully examined in these laboratories and reported recently by Henderson (14).

The lumbar chain from the level of L_1 or L_2 through L_7 (and in several recent animals also including the upper sacral ganglia) was excised on one side only, the chain being left intact on the opposite side so that one of the hind feet may serve as the 'denervated' and the other as the control foot. All observations were made upon the large central pad of the foot (an area comparing with the anterior portion of the plantar surface in man) and closely adjacent areas. Successful operations have been performed and the functional results carefully studied in 11 animals, 6 of which have come to autopsy while the remainder are being studied for more prolonged periods of time.

RESULTS

Figure 2 correlates the vascular, sweating and temperature responses which have been observed in one of our animals. Preoperative blood flows showed wide

variations, quite comparable to those observed in the human palmar and plantar surfaces. The skin pulses were small and the skin temperatures were low when the dog was exposed to a cool environment, and conversely, the pulse was relatively large and the temperatures were high in a warm environment. Spontaneous vasomotor and sudomotor activity was apparent during experiments in a quiet laboratory, and vasoconstrictions were readily induced by the same sort of stimulation that elicits constrictions in man. Sweating responses were more variable and elicited with greater

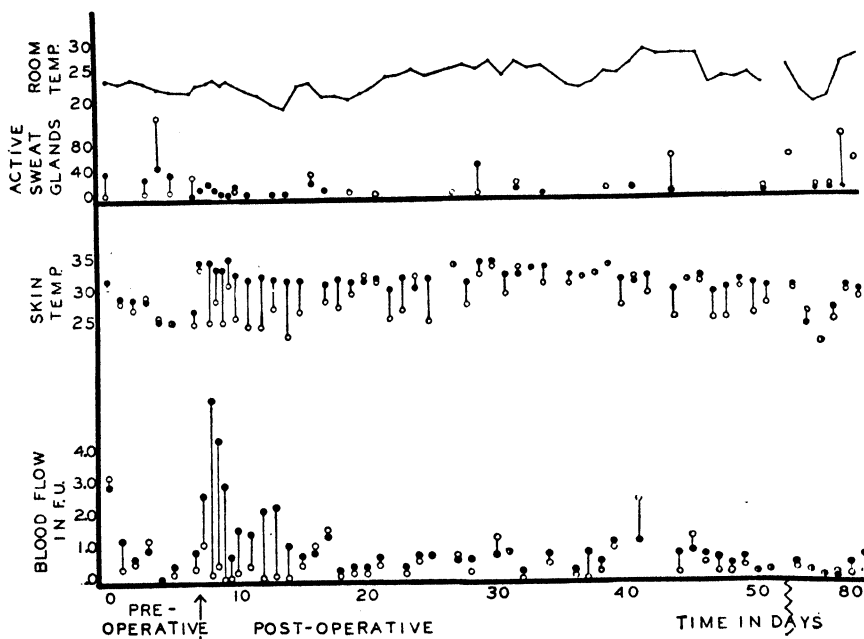


Fig. 2. DAILY OBSERVATIONS of vascular, sweating and skin temperature on the operated (closed circles) and the control (open circles) foot pads during the preoperative and postoperative periods in dog *Ra 12*. At operation (indicated by arrow) the lumbar chain was removed completely from the crus of the diaphragm (L_1) to the lower most lumbar ganglion. The chain on the opposite side was left intact. The ganglionectomy was verified 7 months after operation and the last lumbar and upper sacral ganglia were removed with but little change in results observed.

difficulty, being observed most readily on the junctional tissue at the immediately superior border of the large central pad. Our opinion is that the absence of sweating in our experimental animals was not as significant as the positive demonstration of sweating. In the latter instances definite evidence of an intact innervation seems assured, whereas the absence of sweating in the dog does not necessarily indicate an absence of innervation. Skin temperatures generally followed the gross trends of blood flow alterations, but our experience has been that the temperature response is much slower and lags behind the changes in blood flow to such an extent as to convey misinformation in some instances. This is a particularly important consideration in evaluating vasomotor activity. In all of these representations of sympathetic func-

tion, the responses on the two sides were usually quite comparable during the pre-operative control period.

Immediately after the operation, and while the animal was still under the influence of the anesthetic, blood flow, sweating and skin temperature responses showed considerable variation. The particular anesthetic employed has an important influence, in confirmation of the report of Herrick *et al.* (15), in setting the immediately postoperative levels of blood flow. Under barbiturate anesthesia (sodium pentobarbital in most of our operations) the flows in both operated and control feet were frequently, but not always, equal and were nearly maximal within a few minutes after closing the incision. As the anesthesia decreased, the vessels on the control side progressively constricted while those on the denervated side remained open, at or near the maximal dilatation exhibited during the deepest anesthesia. In some animals, however, the vessels on both sides remained constricted early in the recovery period even under barbiturate anesthesia, and the paws did not show large differences in flow and temperature until the animal had almost completely recovered from the anesthesia.

The blood flow and skin temperature generally remained high in the denervated foot and unusually low in the control foot for 18 to 48 hours. Following the immediately postoperative period of extreme dilatation, an obvious recovery of vascular tone occurred in the denervated foot, and in many instances blood flow in the control foot exceeded that in the denervated foot. This was particularly true when the environmental temperature became elevated. This apparent recovery of vascular tone occurred quickly (within 2-7 days) and although flows on the denervated side usually remained somewhat above the control flows in a cool environment, differences were often small or even insignificant within 4 or 5 days postoperatively and thereafter. The vessels on the denervated side responded to cool environments with a high degree of tone much like that observed on the control side, although typical, sharp vasoconstrictions were absent at this time. This recovery of vascular tone appeared to occur whether the environmental temperature was maintained at a high or low level, since it occurred in both high and low environmental temperatures without significant differences in either series of experiments.

It should be mentioned that in 2 animals there was practically no dilation on the operated side following operation, although records taken both pre- and post-operatively have shown relatively high blood flows when the environmental temperature was elevated. One of these animals has come to autopsy and the completeness of lumbar sympathectomy has been established.

In general, characteristic differences were noted in skin temperatures on the denervated and control side for longer periods than were apparent in the average blood flows. We attempt no explanation for this possible discrepancy but feel secure in the general correspondence and the progressive decline, together with a narrowing of the differences both in flow and in temperature exhibited on the two sides after operation. In all of the animals which we have studied for prolonged periods (up to 10 months at time of writing) the temperature differences observed immediately postoperatively have been greatly diminished or completely abolished after a period of a few weeks or months.

Sweating responses exhibited one of two patterns following operation. In the majority of animals, there was an apparent decline in sweating for the first few days after operation, with definite bilateral responses appearing at variable intervals thereafter. Such responses were repeatedly observed both before and after any possible nerve degeneration might have been expected. In several instances, however, definitely exaggerated sweating responses were observed on the denervated foot with little or no sweating on the control side. This exaggerated output of sweat was shown

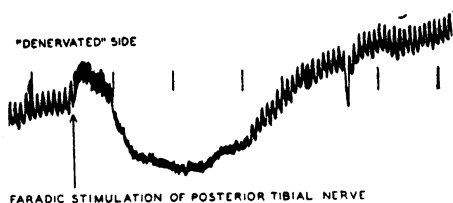


Fig. 3. PROFOUND VASCULAR CONSTRICTION in the foot pad on the operated (denervated) side when strong faradic stimulation was applied to the tibial nerve. Similar constrictions sometimes followed stimulation of the deep peroneal nerve. The signals represent 5-second intervals.

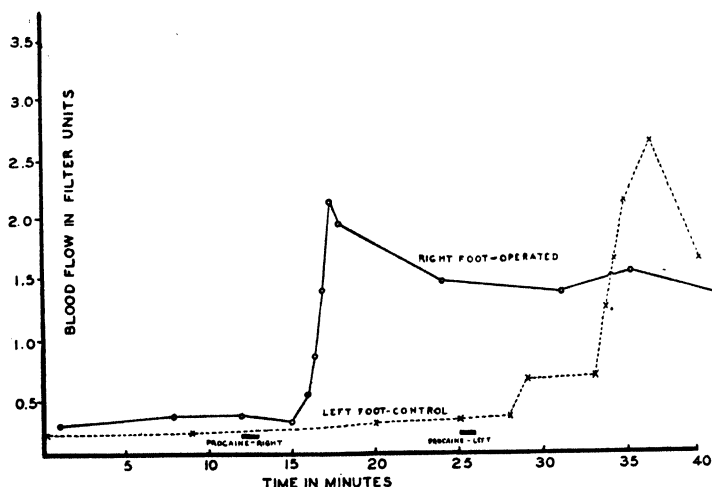


Fig. 4. VASCULAR RELAXATION in the foot pads on both the control and operated sides following infiltration of 2 per cent procaine HCl around the peroneal and tibial nerves.

for one to two weeks and was then followed by the more sporadic sweating patterns observed in the other animals. We should like to emphasize the fact that in both patterns positive though usually reduced sweating responses have been observed postoperatively in all animals.

The recovery of tone in the supposedly denervated blood vessels, the progressive fall in skin temperatures somewhat later but generally coinciding with the decline in blood flow, and the occurrence of definite sweat responses postoperatively, all in the absence of any demonstrable regeneration, lead to the conclusion (similar to that reached by Ray and Console in their series of human patients) that residual pathways exist after complete removal of the lumbar sympathetic trunk has been accom-

plished. Turning therefore to the peripheral nerves we sought definite physiological evidence which would localize these pathways. Application of faradic stimulation to the tibial and in some instances the deep peroneal nerves resulted in positive and sometimes powerful constriction of the blood vessels in both feet. In some instances (fig. 3) the flow through the denervated pad was completely obliterated.

Thus the functional presence of constrictor pathways was demonstrated long after the probable completion of degeneration and before regeneration could have occurred. Although intact sacral ganglia and their postganglionic fibers may have been the route for such pathways, it is improbable that such postganglionic cells initiate a flow of impulses to produce a vasomotor response in the absence of preganglionic connections with the central nervous system. In order to check these possibilities comparable experiments were performed on animals in which the upper sacral chain had been removed along with the lumbar chain; residual vasoconstrictor fibers were still demonstrable in the peripheral nerves by faradic stimulation and by nerve block.

The existence of accessory constrictor pathways was additionally confirmed by observations at various intervals after denervation during blockade of the peripheral nerves in the leg with 2 per cent procaine (fig. 4). A prompt and marked rise in blood flow on both the control and denervated feet indicated the cessation of flow of constrictor impulses by way of these peripheral nerves. This evidence compels the conclusion that accessory sympathetic pathways do exist after anatomically proved complete removal of the lumbar and upper sacral sympathetic trunk. These residual pathways can and do assume some measure of functional control of the blood vessels and sweat glands after extirpation of the above chain.

Serial sections of the spinal nerves, and their intercommunicating rami in the lumbar segments taken from the operated animals at autopsy have thus far revealed ganglion cell aggregates (with some variation from animal to animal) in relation to the second, third, fourth and sixth lumbar nerves. Most of these ganglion cells were imbedded in the ventral primary ramus of the spinal nerve, but none of the aggregates was large enough to be observed macroscopically. Some of these 'ganglia' were located adjacent to the sites of origin of the white communicating rami, others were located more distally. Figure 5 shows the location of the residual ganglion cells found thus far in the dog. In no animal

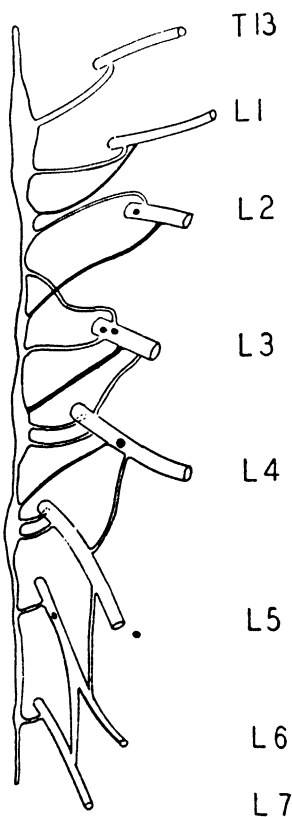


Fig. 5. SUMMARIZATION of the locations of accessory sympathetic ganglion cells in the ventral primary ramus of the lumbar spinal nerves. Also shown are slender communications between the second and third lumbar nerves and successively lower segments.

have cells been found in all of the locations and usually in only one to 3. Cell counts indicated a range from 8 to 56 cells. The largest ganglion observed thus far was located in the third lumbar nerve. An interconnecting ramus occasionally extends from the second to the third lumbar spinal nerves. This was found in 4 of 12 sides dissected. Interconnecting rami exist bilaterally from L_3 through S_2 in the usual pattern of the lumbosacral plexus (fig. 5). Preganglionic fibers will reach these aggregates of ganglion cells via the spinal nerve in which they are located or from a higher level without traversing the sympathetic trunk. A group of ganglion cells located in L_5 or L_6 would probably have to receive their preganglionic connections from L_4 , L_3 or possibly even L_2 , because in so far as is known at the present preganglionic fibers do not arise lower than L_4 (14, 16). Postganglionic sympathetic fibers derived from ganglion cells located in lumbar spinal roots, as well as fibers derived from sympathetic trunk ganglia will also traverse these rami to reach lower levels. Therefore the residual sympathetic activity in the hind limbs, following extirpation of the lumbosacral segments of the sympathetic trunk, is probably due to the postganglionic fibers from these remaining cells.

INTERPRETATION

Complete verified extirpation of the lumbar and upper sacral paravertebral ganglion chain does not eliminate vasomotor control over the blood vessels of the pad of the dog's hind foot. Support for this statement is offered in the observations recorded above: 1) vasoconstrictor reflexes are demonstrable after denervation when regeneration is not a possible explanation. 2) Direct stimulation of the tibial and the deep peroneal nerves on the denervated side elicited constrictions sometimes as marked as those which could be elicited on the control, non-operated side by the same procedure. 3) Local blockade of these nerves by procaine resulted in vasodilatation in the paw's pad both on the control and the denervated sides. The availability of vasoconstrictor pathways by routes other than the paravertebral ganglion chain is thereby demonstrated.

The functional importance of these accessory pathways may be inferred from vasoconstrictor reflexes present after sympathetic trunk extirpation and from the extent of the vascular relaxation which results from their blockade. We believe that the evidence now available to us in this laboratory emphasizes their practical importance in the interpretation of the variable results of sympathectomy. We certainly do not deny, however, the importance of such phenomena as adrenaline sensitization and direct vascular responses to cold.

The determination of how completely a small residuum of fibers can take over the function of the original vasomotor innervation is an intriguing problem. Its possible relation to the processes of sensitization remains for exploration. In addition the possible relationship of a small residuum of fibers to the peripheral neuro-effector mechanism and the extent of its control of autonomically innervated structures should be investigated. The relation of the recovery of vascular tone after denervation to the demonstrated existence of accessory vasoconstrictor pathways is suggested by the effects of nerve block. We are unprepared at the moment to separate either in

time or in importance, so-called inherent tone and neuro-vasoconstrictor tone. Some of our observations suggest the operation of the former in the initial recovery of tone after operation.

Although heat and cold have well-known direct effects on blood vessels (17), the relation of pad flows to environmental temperature in this series of experiments is such as to force attention to a possible role of accessory vasoconstrictor pathways in the reactions to temperature. A close quantitative as well as qualitative parallelism in the vascular status of the control and denervated pads at various environmental temperatures would be difficult to explain if vasoconstrictor fibers are excluded from the mechanism on the operated side. They cannot be excluded on the control side. If we should assign the same role to the accessory pathways on the operated side as we do to the normal vasomotor supply in the reactions to temperature, we would be forced to conclude that this very small residuum of fibers can substitute in large part for the normal more abundant supply. What then makes this possible? We are tempted by the potentialities inherent in the mechanisms of sensitization.

The existence of functionally active vasoconstrictor (and also sudomotor) pathways after complete paravertebral ganglionectomy suggests the importance of the accessory ganglion cells in the anterior roots of the intact animal. Although we are inclined to make this obvious correlation, we must caution that its specific demonstration still remains for exploration.

SUMMARY

Carefully controlled observations in the trained, unanesthetized dog are reported on peripheral vascular, sweating and temperature responses following verified complete removal of the lumbar sympathetic trunk. Precise anatomical and histological analysis of the spinal nerve roots is correlated with the functional studies. Immediately following operation, relatively high blood flow and surface temperatures were usually observed in the pad of the hind paw. Within 2 to 7 days, declining flows indicated a remarkable recovery of vascular tone. Positive though frequently reduced sweating responses were observed in all animals at various intervals following operation.

The existence of accessory vasoconstrictor pathways after a complete lumbar sympathectomy was demonstrated by two series of observations: 1) Direct faradic stimulation of the tibial and peroneal nerves induced strong vasoconstrictions in the foot pads; 2) procaine blockade of these nerves resulted in prompt vascular relaxation with high blood flows. The recovery in tone after operation may be related to these accessory fibers. A possible anatomical basis for these observations may be inferred from the fact that serial sections of the spinal nerves and their intercommunicating rami in the lumbar segments taken from the operated animals at autopsy revealed ganglion cell aggregates (with some variation from animal to animal) in relation to ventral primary divisions of the second, third, fourth and sixth lumbar nerves. The postganglionic fibers of these cells apparently pass directly along the ventral primary ramus of the spinal nerve without entering the paravertebral ganglion chain. They would not be interrupted by lumbar ganglionectomy.

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EFFECT OF SOME DRUGS ON THE POLARIZATION STATE OF SPINAL CORD ELEMENTS¹

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DURING asphyxiation of the spinal cord a potential develops between an electrode located in the gray matter, and an indifferent one placed on a root or on the cord surface (1). The gray matter becomes negative with respect to the external electrode. This potential is believed to indicate the depolarization of the most sensitive part of the neuron (probably the cell body). After a few minutes of cord asphyxiation the 'depolarization' potential reaches a maximum and then starts to decline; this may be the expression of the depolarization of the more resistant parts of the neuron (probably fibers).

With certain restrictions the asphyxial depolarization potential can serve as an indicator of the polarization state of the nervous elements in the spinal cord, since changes in the polarization state will obviously have an influence on this phenomenon. However, the maximum value of the depolarization potential and also the moment at which this maximum is reached will be influenced by variations in the moment at which depolarization begins in the various parts of the neuron, and the rate at which it proceeds (2). Therefore, in using the depolarization potential as an indicator of the polarization state of the cord elements, those experiments have to be excluded in which it is likely that such variations occurred (as indicated by a change in the moment at which the depolarization potential reaches its maximum). With these conditions in mind the effect of ether and sodium pentobarbital on the polarization state of cord elements was previously studied (2). Administration of these narcotics does not cause an appreciable change in the potential between gray matter and indifferent electrode, but decreases the asphyxial depolarization potential markedly. From these observations the conclusion was drawn that ether and sodium pentobarbital cause a uniform depolarization of the neuron. Simultaneous and equal depolarization of the various parts of the neuron cannot be expected to produce a potential difference between gray matter and ventral root, since, as mentioned above, with the method employed only differences in the polarization state of the neuron parts can be detected. Such a uniformly depolarized neuron, however, will produce a reduced asphyxial depolarization potential. With the same method it was shown that nicotine even in large doses does not affect the polarization state of the cord elements (3).

In the present investigation the effect of a number of other drugs has been examined in a similar way.

METHOD

The depolarization potential was led off with an active electrode placed in the gray matter of the spinal cord of cats at the level of L7 or S1, and an indifferent electrode on a ventral root of one of these segments. The potentials were amplified and

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recorded with a coil type galvanometer. For a detailed description of method, see reference (1).

As in previous experiments, curare (Intocostrin, E. R. Squibb & Sons, 0.3 to 0.5 cc/kg.) was used to prevent disturbance of the electrodes by active movements of the animal. This necessitated artificial respiration during the experiment. Many of the drugs in the doses used tend to produce a considerable drop in blood pressure, which may impair the oxygenation of the cord and thus cause asphyxial depolarization. It was thus necessary to record the blood pressure continuously during the experiment and to counteract any tendency of the pressure to drop below 100 mm. Hg by the infusion of epinephrine (10^{-6}) into the jugular vein. The drugs were injected into the carotid artery, reaching the heart after dilution in the peripheral circulation. In this way it was found that larger doses of drugs could be injected faster than by the intravenous route.

Asphyxiation of the cord was produced by occlusion of the aorta with a loop of thick cotton thread placed around this vessel between the diaphragm and the celiac artery. The blood supply through the spinal arteries was arrested by cutting the cord at Th 11 or Th 12.

The mechanical disturbance occasioned by the placement of the active electrode in the gray matter of the spinal cord produces a depolarization from which the cord elements recover in 10 to 15 minutes. This recovery is usually manifest as a slow movement of the galvanometer in a direction indicating decreasing negativity of the gray matter. It is therefore necessary to wait 20 to 30 minutes after placing the electrode. After such a waiting period the preparation may remain in a state of constancy for a considerable time during which cord asphyxiations of 1.5 to 2 minutes' duration repeated with intervals of 8 to 10 minutes produce approximately equal maxima of the depolarization potential, at about the same time after the start of asphyxiation. It was customary in the present experiments to asphyxiate two to three times for 1.5 to 2 minutes to ascertain the constancy of the preparation, then to inject the drug to be investigated, followed again by asphyxiations and eventually by repetition of the drug injection, maintaining intervals of 8 to 10 minutes between asphyxiations. This course has been followed with the majority of the compounds examined. In a few, the nature of the drug necessitated a slightly different procedure. Obviously, in this investigation the only useful preparations were those which were constant over a considerable time and in which no failure of the circulation developed. This necessitated discarding a large number of preparations.

In some instances the effect of a drug on the polarization state was compared with its effect on the spinal reflex activity. To this end the kneejerk was recorded in the right hind leg, using for stimulation an electro-magnetic hammer as described by Johnson (4). The flexion reflex was elicited in the left leg by short faradic stimulations of the *m. peroneus superficialis*. Contractions of the *m. tibialis anticus* were recorded. The stimuli for the kneejerk and flexion reflex were given alternately by means of a set of mechanically driven contacts at intervals of 2.5 to 3 seconds.

RESULTS

Diallylbarbituric Acid (Dial). In the narcotic dose of 50 mg/kg. bodyweight (5), this drug caused a slight reduction of the asphyxial depolarization potential,

which was further depressed by a repetition of the injection (table 1, 1-3). Administration of the drug itself did not cause consistent changes in the potential be

TABLE 1. EFFECT OF DRUGS ON THE ASPHYXIAL DEPOLARIZATION POTENTIAL (IN MV.)

NO.	DRUG USED	CONTROL DEP. POT.		MG/KG.	DEP. POT.	MG/KG.	DEP. POT.	MG/KG.	DEP. POT.
1	Dial	4.2	4.4	50	3.9 (-9%)	50	3.0 (-30%)	50	2.5 (-42%)
2			4.8	50	3.9 (-19%)	50	3.1 (-35%)	50	1.6 (-67%)
3		11.5	11.3	50	10.8 (-5%)	50	6.8 (-40%)	50	4.0 (-65%)
4	Pentothal sodium	4.5	4.3	50	2.1 (-52%)				
5			7.2	50	2.3 (-68%)				
6	Chloral	15.7	16.0	300	8.0 (-50%)				
7			8.8	300	6.3 (-28%)				
8		6.8	7.0	300	2.8 (-59%)				
9		6.1	5.9	500	2.0 (-67%)				
10		7.3	7.2	500	1.7 (-77%)				
11	Avertin	3.7	3.6	100	2.7 (-26%)	100	2.1 (-42%)		
12		16.4	17.2	150	13.2 (-21%)	100	4.8 (-71%)		
13		4.0	4.2	100	3.6 (-12%)	100	3.2 (-22%)	100	2.8 (-32%)
14	Dilantin	4.9	4.9	50	2.2 (-55%)	50	1.6 (-67%)		
15		4.5	4.5	50	2.1 (-53%)				
16		4.3	4.4	50	1.4 (-68%)				
17		8.2	7.8	Alk.	8.6 (+8%)	50	4.8 (-40%)	50	2.6 (-68%)
18		11.0	10.3	Alk.	10.0 (-6%)	50	4.7 (-56%)		
19		5.8	6.0	Alk.	6.2 (+5%)	50	2.8 (-53%)		
20	Propylene glycol	2.2	2.0	3 cc.	2.1 (0%)		2.0 (-5%)		
21		4.3	4.3	3 cc.	4.2 (-2%)		4.0 (-7%)		
22	Mesantoin	7.6	7.8	50	5.4 (-30%)	50	3.2 (-58%)	50	1.8 (-77%)
23		3.3	3.3	50	2.7 (-18%)	50	1.9 (-42%)	50	1.4 (-58%)
24		2.5	2.5	50	2.0 (-20%)	50	1.4 (-44%)		
25		10.0	10.6	50	7.2 (-30%)	50	5.8 (-44%)	50	3.2 (-69%)
26	Morphine SO ₄	10.0	9.7	20	10.7 (+8%)	40		40	10.5 (+6%)
27		4.3	4.3	20	5.0 (+16%)	40	5.0 (+16%)	40	4.9 (+14%)
28	Cocaine HCl	5.0	5.4	10	5.1 (-2%)	40	2.6 (-50%)		
29		8.0	7.8	10	6.3 (-20%)	20	4.0 (-49%)	20	3.3 (-58%)
30		7.8	7.5	30	2.7 (-65%)				
31	Convulsant barbiturate	8.8	8.5	5	7.5 (-13%)	50	3.8 (-56%)		3.5 (-60%)
32		8.2	8.6	5	7.4 (-12%)	25	5.8 (-31%)		
33		7.0	7.4	5	5.6 (-22%)	25	4.6 (-36%)		

tween gray matter and indifferent electrode. The effect of diallylbarbituric acid thus seems to be similar to that of sodium pentobarbital, namely uniform depolarization of the spinal neuron.

Sodium Ethyl (1-Methyl-Butyl) Thiobarbiturate (Pentothal Sodium). This drug is characterized by its short action, which makes it feasible to study the recovery of the cord. Pentothal in a dose of 50 mg/kg. produced a marked reduction of the asphyxial depolarization potential (table 1, 4, 5). In figure 1 (*upper record*) the depolarization potential 3 minutes after the start of the pentothal injection is reduced about 60 per cent, but 10 minutes later it has grown again considerably. This record shows further that the administration of the drug does not produce a significant movement of the galvanometer (the small quick deflections are caused by handling the preparation during the injection), indicating that this narcotic also depolarizes the spinal neuron uniformly. Figure 2 shows that the depolarization potential recovers completely about 30 minutes after administration of pentothal (50 mg/kg. bodyweight).

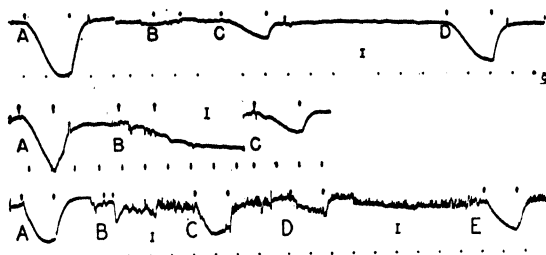


Fig. 1. *Upper record.* Effect of pentothal sodium. A, asphyxial depolarization potential before injection. B, injection of 50 mg. of pentothal/kg. bodyweight. C, asphyxial depolarization potential about 2 minutes after end of injection. D, the same 10 minutes later. The record is continuous. *Middle record.* Effect of chloral. A, asphyxial depolarization potential before injection. B, injection of 500

mg. of chloral/kg. bodyweight. C, depolarization potential recorded 7 minutes after B. *Lower record.* Effect of strychnine. A, asphyxial depolarization potential before strychnine injection. B, injection of 5 mg. of strychnine/kg. bodyweight. C, depolarization potential. Between C and D 20 mg. of strychnine/kg. bodyweight were injected. D and E, asphyxial depolarization potentials recorded with a 9-minute interval. The lower signal indicates in all records time in minutes; the upper signal, beginning and ending of asphyxiations and injections. The vertical line indicates one mv.

Chloral. Chloral, which in a dose of 300 to 500 mg/kg. produces deep narcosis (5), caused a marked depression of the asphyxial depolarization potential (table 1, 6-10). In contrast with the drugs described above, injection of chloral caused consistently a galvanometer movement indicating increasing negativity of the gray matter (fig. 1, *middle record*). This observation indicates that chloral does not affect the spinal neuron uniformly but that the parts in the gray matter (probably the cells) are depolarized to a greater extent than those in the white matter.

Tribromethanol (Avertin). The effect of chloral, which did not seem to affect the neuron uniformly, induced the examination of other halogen-substituted narcotics. Tribromethanol, in the narcotic dose of 100 to 150 mg/kg. (5), caused a moderate reduction of the asphyxial depolarization potential (table 1, 11-13). In contrast to chloral, administration of tribromethanol did not consistently produce negativity of the gray matter; it therefore seems to affect the neuron uniformly.

Chloroform. Also a halogen-substituted narcotic, this drug administered for 5 minutes in the respiratory air caused a severe depression of the asphyxial depolarization potential which was readily reversible (table 2). It has been possible to suppress the asphyxial depolarization potential completely with ether (2). Attempts to do the

same with chloroform resulted in damage to the circulation and a drop in blood pressure. During administration of chloroform no consistent changes of the potential between gray matter and external electrode developed in those preparations in which the blood pressure could be maintained (uniform depolarization of the neuron).

Diphenyl Hydantoin (Dilantin). This is a compound with marked anticonvulsant activity but devoid of a pronounced hypnotic action. Its injection in a dose of 50 mg/kg. bodyweight, a dose used in experimental work on the anticonvulsive properties of this compound (6), caused a marked depression of the depolarization potential (table 1, 14-19). Dilantin is water soluble at pH 11 and the drug therefore had to be injected as a strongly alkaline solution. The same amount of alkali used for dissolving the drug was injected in a number of preparations (table 1, 17-19). This was without consistent effect on the asphyxial depolarization potential.

Administration of diphenyl hydantoin itself had no effect on the position of the galvanometer (uniform depolarization of the neuron).

Fig. 2. DEPOLARIZATION AND RECOVERY, after the injection of 50 mg. of pentothal sodium/kg. bodyweight. Injection at P. Abscissa, time in minutes; ordinate, asphyxial depolarization potential in mv.

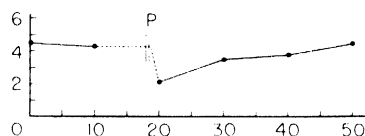


TABLE 2. EFFECT OF CHLOROFORM ON ASPHYXIAL DEPOLARIZATION POTENTIAL (IN MV.)

NO.	BEFORE CHLOROFORM ADMINISTRATION	AFTER 5 MIN. CHLOROFORM ADMINISTRATION	10 MIN. AFTER END OF CHLOROFORM ADMINISTRATION
1	9.0	3.3 (-63%)	7.7 (-14%)
2	12.3	3.7 (-70%)	10.8 (-12%)
3	6.3	3.7 (-41%)	6.2 (-2%)

In some experiments the effect of dilantin on the spinal reflex activity was investigated. Injection of 50 mg. kg. bodyweight during a series of reflex contractions had very little effect (fig. 3, I). In a few experiments the kneejerk contractions increased slightly after the injection. Since this was also observed after injection of alkali only, the enhancement may be due to the alkalinity of the solution. The injection of 50 mg. of pentobarbital kg. stopped the kneejerk completely.

Methyl-Phenyl-Ethyl Hydantoin (Mesantoin). Another anticonvulsant, this drug has even more unfavorable solubility properties than diphenyl hydantoin. Under gentle heating (60°C.) it is soluble up to a concentration of about 50 mg/cc. in propylene glycol. In 2 experiments 3 cc. of propylene glycol/kg. bodyweight was injected (table 1, 20, 21), which caused slight depressions of the asphyxial depolarization potential. Injection of 50 mg. of methyl-phenyl-ethyl hydantoin (in one cc. of propylene glycol) per kg. bodyweight, caused a considerably greater depression of the potential (table 1, 22-25). The injection itself had no consistent effect, indicating that this drug affects the spinal neuron uniformly.

The effect was examined of the administration of propylene glycol and of methyl-phenyl-ethyl hydantoin dissolved in this compound on the kneejerk and

flexion reflex. Administration of propylene glycol (3 cc/kg. bodyweight) had no effect. Also very little effect was observed from injection of 50 mg/kg. of mesantoin even when this was repeated (fig. 3, II). Sodium pentobarbital in a dose of 75 mg/kg. bodyweight stopped the kneejerk promptly.

Morphine Sulfate. Administered in large doses (up to 100 mg/kg. bodyweight) morphine sulfate did not cause a decrease of the asphyxial depolarization potential; on the contrary it caused a moderate increase of the potential (table 1, 26, 27). The injection of the drug itself had no consistent effect.

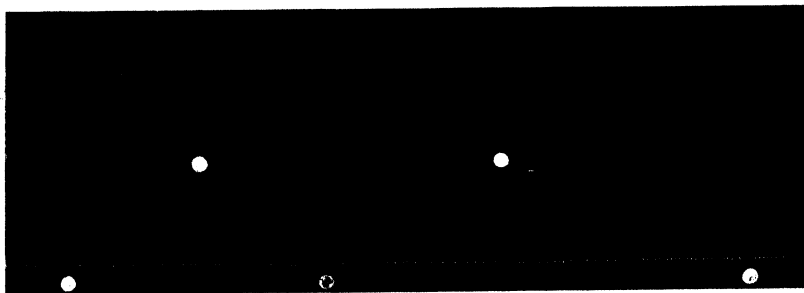
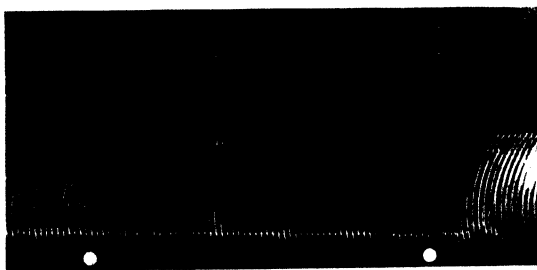


Fig. 3. I, effect of *dilantin* on the kneejerk. At the first dot 50 mg. of *dilantin*/kg. bodyweight were injected; at the second dot, 50 mg. of sodium pentobarbital/kg. bodyweight. II, effect of *mesantoin* on the kneejerk. At the first dot 50 mg. of *mesantoin*/kg. bodyweight were injected; at the second dot this was repeated. At the third dot 75 mg. of sodium pentobarbital/kg. bodyweight were administered.

Fig. 4. ANTAGONISM BETWEEN SODIUM PENTOBARBITAL AND STRYCHNINE. *Upper record* is of the flexion reflex, *lower record* of the kneejerk. I, at the dot, 75 mg. of pentobarbital/kg. bodyweight were injected. II, 20 mg. of strychnine nitrate/kg. bodyweight restored kneejerk and flexion reflex.



Cocaine Hydrochloride. In a dose of 10 mg/kg. bodyweight cocaine hydrochloride caused a slight reduction of the asphyxial depolarization potential; additional doses further depressed this potential (table 1, 28-30). The injection itself did not produce consistent changes in the galvanometer position.

Administration of 10 mg. of cocaine/kg. bodyweight enhanced the kneejerk contractions somewhat, but amounts larger than 20 mg/kg. caused a decrease.

Strychnine Nitrate. This alkaloid caused the appearance of more or less pronounced, continual irregularities of the base line (fig. 1, *lower record A, B and C*). Before the injection (5 mg/kg. bodyweight) the record was smooth except for some disturbances caused by handling the preparation during clamping and releasing the aorta. The irregularity of the base line after the injection remained for the rest of the experiment. Care was taken to curarize the preparation completely, to avoid re-

cording the mechanical and electrical disturbances caused by convulsive muscle contraction. It seems therefore that the irregularity of the base line is a spinal phenomenon caused by bursts of activity of the spinal neurons, which produce the strychnine convulsions in the non-curarized preparation. This is supported by the observation that deep narcosis abolishes the irregularities of the base line more or less completely, as well as suppresses the convulsions in the non-curarized animal.

Strychnine in doses between 5 and 25 mg/kg. bodyweight may cause a depression of the asphyxial depolarization potential (fig. 1, *lower record A, B and C*). There was, however, no direct relation between the dose of the drug and the depth of the depression, as was generally found for the drugs described above. For instance in one experiment the control values of the asphyxial depolarization potential were 6.1 and 6.2 mv. Injection of 5 mg. of strychnine/kg. bodyweight caused marked irregularities of the base line. The asphyxial depolarization potential recorded a few minutes after the injection was only 2.6 mv. Then 20 mg/kg. of the drug was administered and after a few minutes a depolarization potential of 6.4 mv. was recorded; after another injection of 20 mg/kg. the asphyxial depolarization potential was 6.0 mv. Often the asphyxial depolarization potential was depressed most severely shortly after injection of the drug, and subsequent asphyxiations of the cord produced larger or even pre-injection potentials. Figure 1 (*lower record*) shows this variability: the first depolarization potential (*D*), after the injection of 25 mg. of strychnine/kg. bodyweight, is considerably depressed, 9 minutes later a much larger potential (*E*) is recorded. There is another difference between these 2 depolarization potentials: the base line irregularities continue during the first asphyxiation, but are about absent during the second one. It has been observed generally that the asphyxial depolarization potential is depressed in the presence of strychnine-induced irregularities of the base line.

During the convulsive discharge, numerous spinal neurons will conduct impulses at any moment. The conducted impulse spreads within a very short time over that part of the neuron which is so near the active electrode that its potential changes will be recorded. After this the neuron remains for a moment in the excited state, during which the conducting membrane is depolarized or even generates a potential in the reverse direction (7). With the methods used this constitutes statistically a partial uniform depolarization of the group of neurons from which it led off. Asphyxiations during a convulsive discharge thus can be expected to produce a reduced depolarization potential. This concept accounts for the varying effect of strychnine on the depolarization potential, since the depression would depend on the convulsive activity of the cord which may vary greatly from moment to moment. It seems likely that strychnine has no pronounced effect on the polarization state of the spinal cord elements other than that caused by the convulsive discharge.

There exists an antagonism between the action of the narcotics and of strychnine. Figure 4 shows that the flexion reflex is totally abolished and the kneejerk greatly reduced by sodium pentobarbital in a dose of 75 mg/kg. bodyweight. Injection of 20 mg. of strychnine nitrate/kg. bodyweight restores these reflexes. It seemed of interest to investigate the effect of such doses of pentobarbital and strychnine on the depolarization potential. In table 3 two such experiments are recorded.

The asphyxial depolarization potential reduced by the injection of 75 mg. sodium pentobarbital/kg. bodyweight decreased even more after the injection of 20 mg. of strychnine nitrate/kg. The latter decline of the depolarization potential may be caused by an activity of a convulsive type evoked by the alkaloid, as in the non-narcotized preparation.

*Sodium (1,3-Dimethylbutyl) Ethyl Barbiturate*³. This is a barbiturate with a convulsant instead of hypnotic action (8-10). In convulsive doses (5 mg/kg.) this compound produced a moderate reduction of the asphyxial depolarization potential (table 1, 31-33). In some instances the injection of the compound caused a slight irregularity of the base line like that observed more clearly after administration of strychnine. It seems likely that these irregularities are caused by the convulsive discharge. The decline of the asphyxial depolarization potential by such a small dose of sodium (1,3-dimethylbutyl) ethyl barbiturate may be partly or completely due to the convulsive activity of the cord, by the mechanism discussed for strychnine. However, injection of additional doses of this compound (25 and in one case 50 mg/kg.) caused invariably a more marked decline of the depolarization potential, which

TABLE 3. EFFECT OF SODIUM PENTOBARBITAL AND STRYCHNINE ON THE ASPHYXIAL DEPOLARIZATION POTENTIAL (IN MV.)

POTENTIAL (IN MV.)						
NO.	CONTROL DEF. POT.		PENTOBARBITAL 75 MG/KG.		STRYCHENINE NITRATE 10 MG/KG.	
1	8.8	9.0	4.0(-55%)	3.8(-57%)	2.8(-69%)	2.6(-71%)
2	12.1	12.1	4.8(-64%)	4.3(-66%)	4.0(-67%)	3.8(-69%)

may indicate that this barbiturate has a depolarizing action like the others examined. The administration of this compound had no effect on the galvanometer position.

DISCUSSION

In a previous paper (2) the possibility has been considered that depolarization of the cord elements contributes to the narcotic action of sodium pentobarbital and ether. The depolarization would cause a decrease of the presynaptic potential which in the electrical concept of synaptic conduction is the transmitter across the synapse. This is not the only effect of the narcotics, since ether has been found to raise the threshold of excitation of peripheral nerve (11). Furthermore Brooks and Eccles (12) observed in the spinal motoneuron that increasing amounts of sodium pentobarbital first delayed, and finally prevented the setting up of a conducted impulse by the synaptic potential (stabilizing effect on the membrane). The depressing action of the narcotics on the presynaptic potential and the effect on the postsynaptic membrane described by Brooks and Eccles would at the narcotic concentration of the drug stop synaptic transmission.

The present series of experiments was set up to determine the importance of depolarization of cord elements for the narcotic action of drugs. Obviously, if depolarization were the determining factor for the action of a narcotic, a high cor-

³ We are indebted to Dr. Gordon A. Alles for kindly supplying us with this compound.

relation between these two effects would be expected. Many narcotics, like diallyl-barbituric acid, sodium ethyl (1-methylbutyl) thiobarbiturate, chloral, chloroform and tribromethanol, cause a depression of the depolarization potential. However, the narcotic doses of these drugs, producing comparable states of central nervous depression, cause quite a varying degree of depression of the asphyxial depolarization potential (from 5 to 20 per cent as in the case of diallylbarbituric acid, to 30 to 60 per cent as in the case of chloral). The anticonvulsants, diphenyl hydantoin and methyl-phenyl-ethyl hydantoin, which have little effect on the kneejerk and flexion reflex in a dose of 50 mg/kg. bodyweight, cause a pronounced depression of the asphyxial depolarization potential. Cocaine, which in doses up to 20 mg/kg. bodyweight did not depress but slightly enhanced the spinal reflexes, caused a depression of the depolarization potential. A complete lack of parallel between synaptic conduction and depression of the depolarization potential was found in the antagonism between sodium pentobarbital and strychnine. The recovery of the reflex activity, lost after the injection of an adequate dose of sodium pentobarbital by the administration of strychnine, was in no case accompanied by an increase of the asphyxial depolarization potential. The observations, especially with the anticonvulsants and with strychnine, show that synaptic conduction is not incompatible with a considerable uniform depolarization of the spinal neuron.

The depolarizing and membrane stabilizing effects of narcotics are not directly related as shown by observations on peripheral nerve. Bishop (13) noted that conduction in peripheral nerve can be stopped with cocaine and amyl alcohol without depolarization, even with an increase of the membrane potential. Similar results were obtained by Bennett and Chinburg (14) with a number of local anesthetics. Crescitelli (15) working with carbamates was able to stop conduction in peripheral nerve not only without depolarization but with hyperpolarization. On the other hand, the arrest of conduction by ether and alcohol in peripheral nerve seems always to be accompanied by considerable depolarization (11, 16, 17). Both membrane stabilization and depolarization may, when sufficiently pronounced, stop conduction in the nerve and central nervous system. It seems that the effect of some compounds (cocaine and carbamates) on peripheral nerve is due exclusively to the membrane stabilizing action. Ether and alcohol may interrupt conduction mainly by depolarization, since Lorente de Nó (16) and Gallego (17) could restore conduction by repolarizing the membrane with an externally applied potential. All narcotics examined have been found to have a depolarizing effect on the central nervous elements. However, the majority of these narcotics had a relatively weak depolarizing action in narcotic dose. It seems likely that in the action of these compounds depolarization is of minor importance. Ether, however, is a potent depolarizing agent for the central (2) as well as for the peripheral (16, 11) nervous system, and it is possible that the action of this narcotic on the central nervous system depends to a considerable extent on its depolarizing effect. The anticonvulsants investigated produced considerable depolarization of the spinal cord elements, but since they do not depress synaptic conduction, their membrane stabilizing effect must be weak or even absent. It seems possible that the anticonvulsants derive their useful properties from this combination of effects on the central nervous system.

Crescitelli (15) regards depolarization caused by higher concentrations of carbamate as a sign of toxic damage to the nerve, which is supported by his observation that such depolarization may become irreversible. In the present experiments, narcotics administered in narcotic dose have in general produced depolarization of the spinal cord elements. The reversibility of the effects of such doses of narcotics is obviously well established and in the experiments in which recovery was studied (sodium ethyl (1-methylbutyl) thiobarbiturate) a prompt reversibility was found. It therefore seems that in these instances the depolarization is not caused by damage to the spinal neuron, but is part of the pharmacological action of the drug.

It is interesting that whereas Bishop observed (13) hyperpolarization by cocaine in anesthetic concentration, the first effect of cocaine on the spinal cord was depolarization and reflex hyperexcitability. The cocaine concentration used in the present experiments (10 to 50 mg/kg. bodyweight) is lower than that used by Bishop to produce the hyperpolarization (1 to 5×10^{-4}). Lorente de N6 (16) found evidence for a depressing effect of cocaine on the metabolic processes related to the maintenance of the membrane potential, and concluded that cocaine poisoning of a nerve affects the polarization state only in those instances in which the value of the membrane potential is dependent upon a rapid oxygen consumption by the nerve (as during repolarization after anoxia). It seems possible that the difference of the effect of cocaine on the polarization state of peripheral and central nervous tissue is related to metabolic differences in these tissues.

SUMMARY

The effect of a number of drugs on the polarization state of cord elements was investigated. Most of the narcotics investigated (ether, sodium pentobarbital, dial, pentothal, avertin, chloroform) caused a uniform depolarization of the spinal neuron. Chloral seems to depolarize the nerve cell more than the fibers. The anticonvulsants, dilantin and mesantoin, produced considerable uniform depolarization in doses which did not affect kneejerk and flexion reflex. Of the alkaloids examined, cocaine produced a uniform depolarization. Morphine and strychnine did not depolarize the spinal cord elements. The strychnine convulsions, however, produce a functional depolarization. A barbiturate with convulsive properties (sodium 1,3-dimethylbutyl ethyl barbiturate) probably caused functional depolarization due to the convulsive discharges, but depolarized in higher concentrations like the narcotic barbiturates. The phenomena observed are discussed in relation to the pharmacological actions of the drugs investigated.

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FORMATION OF CONDITIONED AVOIDANCE RESPONSES IN YOUNG PUPPIES

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THE dog has been, since the time of Pavlov, a classical subject for the demonstration of conditioned reflexes. However, in the extensive bibliography found in Pavlov's lectures on reflexes (1) there is no account of any study of a conditioning process in very young animals. Members of the Russian school have studied this process in other species. Golubeva (2) reported that conditioned motor reactions to acoustic stimuli could be readily formed by newborn guinea pigs. Kasatkin and Levikova (3) state that visually initiated conditioned reflexes in the human infant can be formed at the ages of 6 to 8 weeks and visual discriminatory reactions may be formed by the fourth month. D. P. Marquis (4) found that conditioned responses could be formed in infants at less than 10 days of age. Wickens and Wickens (5) were unable to obtain clear-cut conditioning in the neonatal human. In the face of such contradictory evidence it seems to be of interest to investigate the possibility of conditioned reflex formation in the newborn puppy and to determine the age at which conditioned reflex formation is possible.

The puppy is not an ideal animal in which to investigate the process of conditioning. A state of quiet alertness is considered to be essential for satisfactory training, but a newborn puppy alternates between sleeping and a state of vigorous activity accompanied by loud whining. This agitated state is readily produced by contact with cool surfaces, by hunger and by nocuous stimuli. The disturbance produced by removing a puppy from its littermates for experimental observations generally lasts for several minutes. Any restraining harness or attachment of apparatus for recording of responses evokes such a vigorous response that conditioning is impossible. After several attempts to secure objective recording of the reactions it was decided that the disturbance of the subjects produced by these methods precluded their use, and that greater reliability would be obtained from direct observations, checked always by two or more independent observers.

METHODS

The subjects for this experiment were 25 puppies from 6 litters. *Numbers 1 to 6* were Cocker Spaniels; *7 to 9* and *19 to 22*, Shetland Sheepdogs; *10 to 14*, Wirehaired Foxterriers; *16 to 18*, Springer Spaniels; and *23 to 28*, Kerry Blue Terrier X Beagle hybrids. The first training was given in all cases between the first and fifth day of age. For each test the puppy was removed to a cloth-lined box, cloth strips soaked in salt solution were tied around each fore limb and attached to leads from an in-

duction coil. For those puppies which received shocks the current strength was adjusted gradually above the threshold so as to give a clear response. The conditioned stimuli employed were 1) the sound of a buzzer, 2) the light from a 75-watt bulb placed about one foot above the puppy, and 3) the odor of Karo or Chaperone¹ presented on a glass rod. In each set of experiments control animals were used for observation of the response to the presentation of the conditioned stimulus alone.

The response of puppies of this age to shock alone consists of a generalized flexion of all limbs, whining, raising the head, and attempts to crawl out of the box. None of the puppies ever showed a discrete limb withdrawal, which is readily elicited in most older dogs (6). The vigor of the unconditioned response varies greatly according to the individual's physiological state. At times a barely perceptible wincing is the only response, while at other times whining and vigorous activity persist for periods of a half minute or more. The conditioned response is not exactly the same as the unconditioned. It may best be called an 'alerting reaction.' The animal raises his head, extends his limbs rigidly and appears to brace himself for the ensuing shock. This difference is clear enough so that a trained observer can readily distinguish these responses from spontaneous activity which may chance to coincide with a presentation of the conditioned stimulus. For each experimental subject the schedule for each day consisted of 10 trials given at one-minute intervals. The first 4 trials consisted of presentation of the conditioned stimulus for 2 seconds followed by a one-second shock. The fifth presentation was conditioned stimulus alone. Trials 6 through 9 were again paired and trial 10 was conditioned stimulus alone.

In each case the nature and vigor of the responses and the degree of alertness of the animal at the time of stimulation were noted. The results of these experiments are recorded in table 1. For each experimental individual the time sequence for the development of the conditioned reflex is very similar. Before the age of 18 days there is no reliable evidence of conditioning to light or sound. Between 18 and 20 days there is scattered evidence of conditioned reflexes but they are poorly defined and occur only sporadically. From 20 days onward conditioned reflexes are definite and highly predictable. It is probable that conditioning to Chaperone and Karo began about 3 days earlier than that to light and sound but stable conditioning was not attained until the 20th day. The very young puppy shows an avoidance response to Chaperone, which somewhat resembles the alerting reaction described above, so that some of the responses recorded as conditioned responses may not actually have been so.

The importance of maturation rather than continued experience is shown by the results on the control puppies. As is indicated in table 1 (*subjects 13, 14, 25, 28*) conditioning occurred within less than 10 trials when the paired stimuli were presented to puppies over 20 days of age.

An attempt was made to condition 8 puppies (nos. 15-22) to the contact of a camel's hair brush stroked four times on the back of the head just before shock was administered. No evidence of conditioning was found. Because of the activity of the puppies, contacts of the back of the head with the sides of the experimental box

¹ Chaperone is a commercial dog repellent, the active ingredient of which is a derivative of citronella.

TABLE 1. AGE OF FORMATION OF CONDITIONED REFLEX (ALERTING RESPONSE) IN NEWBORN PUPPIES

NO.	SEX	CONDITIONED STIMULUS	SHOCK OR CONTROL	RESULTS ¹
1	M	Buzzer	Shock	CR at 20 to 23 days
3	M	Buzzer	Control	'Alerting' at 29 days. Less marked than in exper. animal
4	M	Light	Control	Alerting on 20th day
5	F	Light	Shock	Alerting on 20th day; not clearly different from control, light probably too strong
7	M	Buzzer	Shock	CR at 18-19 days
9	M	Buzzer	Control	Did not develop 'alerting response'; ears pricked up at 20th day when buzzer sounded
10	M	Light	Shock	CR at 21 days
11	M	Light	Shock	CR at 21 days
12	F	Light	Shock	CR at 22 days
13	F	Light	Control	No 'alerting' at 23 days; training started on 23rd day and CR obtained on trial 10
14	M	None	Shock	No 'alerting' at 23 days; training started on 23rd day and CR to light obtained on trial 11
15-18	2 M 2 F	Touch	2 Shock 2 Control	No 'alerting' by 25th day; <i>no. 15</i> conditioned to buzzer in 8 trials on day 25
19-22	1 M 3 F	Touch	2 Shock 2 Control	No 'alerting' by 24th day
23	M	Karo	Shock	CR at 14-21 days; on day 27 CR to Chaperone established in 7 trials
24	F	Karo	Shock	CR at 15-21 days; on day 27 CR to Chaperone established in 7 trials
25	M	Karo	Control	No 'alerting' at 27 days; on day 27 CR to Chaperone established in 7 trials
26	M	Chaperone	Shock	CR at 13-19 days; on day 27 CR to Karo established in 8 trials
27	F	Chaperone	Shock	CR at 15-20 days; on day 27 CR to Karo established in 8 trials
28	F	Chaperone	Control	No 'alerting' at 27 days; on day 27 CR to Karo established in 8 trials

¹ When two days are noted for the appearance of CR's, the first indicates sporadic or questionable responses, the second is the day at which 8 or more of the 10 trials resulted in a CR.

were frequent, and the tactile stimulus was probably not differentiated from accidental stimulations.

DISCUSSION

The above experiments demonstrate that under the experimental conditions used stable conditioned reflexes cannot be formed in the newborn puppy but are readily formed in puppies over 20 days of age. This dependence upon maturation does not seem to be a function of the age in which the sense organs become functional. Dogs at birth respond to odors². The eyes open from 11 to 19 days of age (9), and the auditory meatus at 18 days of age or later. The age of 20 days corresponds to a marked change in the organization of the puppy's reflexes (8) and marks the beginning of the 'critical period' of social development which has been described by Scott (9). All of these experiments seem to indicate that a fundamental change in neural organization of the puppy occurs during the latter part of the third post-natal week.

It should not be concluded from these experiments that it is impossible to produce stable conditioned reflexes in puppies before the age of 20 days. Although there appears to be no correlation between the age at which *stable* reflexes are formed and the particular sensory gateway through which the conditioned stimulus enters, it is possible that the conditionability of the other motor patterns such as suckling may differ from that of the avoidance reaction. It is also possible that stable conditioning could be obtained by more intensive training; as for example, by 100 trials per day rather than 10. The possibility has been considered that our 'alerting reaction' does not represent true conditioning, but that the shocks function merely to raise the general level of alertness so that the animal attends more definitely to any stimulus. Control animals to which shocks were given at random to maintain alertness did not, however, show this exaggerated response, nor did puppies trained with one type of conditioned stimulus give the alerting reaction when other types of stimuli were presented. The results can be best interpreted in terms of maturational change, which probably involves the development of cerebral control over the motor centers.

SUMMARY

Twenty-five newborn puppies have been tested for conditioned avoidance responses to electric shock applied to the forelegs, using sound, light, odor and contact as conditioned stimuli. Receptors for odor and contact appear to function at birth, for light at about 16 days, and for sound at about 19 days. However, the age at which stable CR's developed to sound, light and odorous stimuli was from 18 to 21 days in all cases. Control puppies, not subjected to the conditioning procedure until 20 days of age, form CR's readily when training is commenced. CR's to contact were not produced by our technique. The time of appearance of conditionability corresponds to the beginning of the 'critical period' of social development postulated by Scott, and to the changes in reflex patterns described by Bahrs.

² The possibility of trigeminal stimulus by Karo and Chaperone has not been ruled out on these experiments (7).

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PILOCARPINE SENSITIZATION IN THE PARASYMPATHETICALLY DENERVATED PUPIL OF THE CAT¹

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ANDERSON (1) reported that after removal of the ciliary ganglion the cat's iris immediately becomes sensitized to pilocarpine, instilled into the conjunctival sac. According to this investigator the degree of sensitization does not change with the passage of time. Shen and Cannon (2) observed maximal sensitization to instilled acetylcholine in the eserinated, parasympathetically denervated cat's eye 24 hours after excision of the ciliary ganglion. Keil and Root (3) stated that the response of the parasympathetically denervated cat's eye to intravenously administered acetylcholine does not become maximal until 5 to 7 days after operation. In the present investigation the response of the cat's pupil to topically applied and intravenously administered pilocarpine has been studied at various times after ciliary ganglionectomy.

PROCEDURE

Thirty-six cats were anesthetized by the intraperitoneal injection of sodium pentobarbital and the ciliary ganglion of one side removed under aseptic conditions (2). In 14 of these animals the opposite ciliary ganglia were excised a number of days later. Corneal injury was avoided by placing adhesive tape over the operated eye for the duration of the anesthesia and the operations were performed without noticeable damage to the orbital vessels or extrinsic ocular muscles. Dilation of the pupil occurred at the time of extirpation of the ganglion, but this was not maximal until 24 to 48 hours later.

On various days after the operation, experiments were carried out in a darkened room so that, at the outset, the normal pupil was widely dilated and of approximately the same size as the denervated pupil. Pilocarpine was administered to the unanesthetized animal by intravenous injection into the femoral vein or by instillation into the conjunctival sac. Changes in the horizontal diameters of normal and denervated pupils were measured with a millimeter ruler. With the doses of pilocarpine administered by either route the normal eye did not change in size and was used for a control in the experiments performed on unilaterally ganglionectomized preparations.

When the drug was injected intravenously 0.4 mg. of pilocarpine nitrate (Mal-linckrodt) per kilogram of body weight was used. This quantity was considered to

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represent an optimum dose, since smaller doses failed to evoke maximal constriction of the parasympathetically denervated sphincter and larger doses, up to 1 mg/kg., produced no greater response.

The topical application of pilocarpine in doses corresponding to those used by Anderson (1), namely 0.4 mg. per eye, produced intense and prolonged constriction of the the denervated pupil. When the dose was reduced to 0.04 mg. per eye, the constriction was as intense as that found with the larger dose but it did not last for as long a time. In most of the experiments 0.04 mg. was used, but in certain animals the dose was reduced to 0.02 mg. or even 0.01 mg. per eye.

RESULTS

Latent Period. The response of the parasympathetically denervated pupil to intravenously administered pilocarpine was apparent usually before the injection had

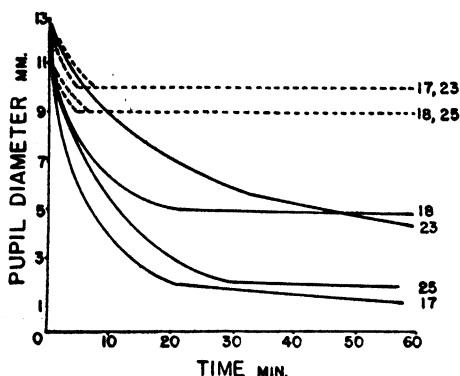


Fig. 1. EFFECT OF INTRAVENOUSLY INJECTED PILOCARPINE (0.4 mg/kg. body weight) upon size of right pupils of cats 17, 18, 23, and 25 on the 2nd (dotted lines) and 6th to 8th days (solid lines) after excision of the ciliary ganglion.

been completed. Miosis of the pupil generally increased for 10 to 20 minutes and was maintained at a constant degree of constriction until at least 45 minutes after the time of injection. Gradual dilatation of the pupil then began and the pupil returned to the control size from 3 to 24 hours after the injection of the drug. When pilocarpine was given topically constriction became apparent in 8 to 20 minutes. The latent period was not influenced by the amount of pilocarpine administered. Miosis lasted for 3 days when 0.04 mg. per eye was given topically and sometimes for as long as 24 hours when the dosage was reduced to 0.01 mg.

Time Required for Maximum Sensitization. In 11 animals, pilocarpine was given intravenously (0.4 mg/kg.) on the first several days after removal of the ciliary ganglion. In every instance, a measurable response of the denervated pupil occurred, but the constriction was never as great as that obtained at a somewhat later date. Figure 1 illustrates the response of 4 cats to pilocarpine given by vein on the second day after operation, and on the 6th to 8th days. Figure 2 is a reproduction of photographs showing that the response of the denervated pupil to equal doses of pilocarpine administered systemically was greater on the 6th than on the 2nd day after denervation. The results indicate that maximal sensitization of the denervated pupil

to intravenously administered pilocarpine occurs 5 to 8 days following extirpation of the ciliary ganglion.

In 7 cats, 0.04 mg. of pilocarpine nitrate, or one-tenth of the dose used by Anderson (1), was placed into the conjunctival sac during the first 5 days after ciliary ganglionectomy. Maximal constriction of the denervated pupil occurred in every instance. When 0.02 mg. of pilocarpine was instilled, maximal constriction of the denervated pupils of 2 cats was observed on the second day after operation, although in neither animal were the sphincters maximally sensitized to pilocarpine given by

Fig. 2. EFFECT OF INTRAVENOUSLY INJECTED PILOCARPINE (0.4 mg/kg. body weight) upon size of right pupil of cat 25, 2, 6, and 13 days after excision of the ciliary ganglion. Note the appearance of maximal sensitivity on the 6th day and the decline in responsiveness on the 13th day.

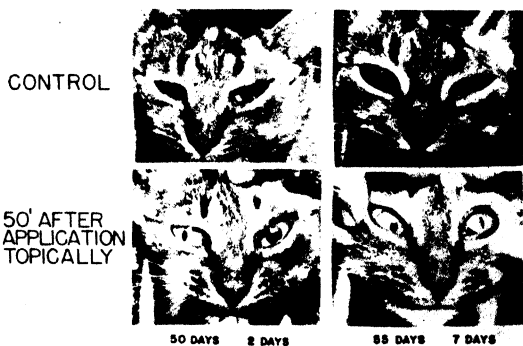
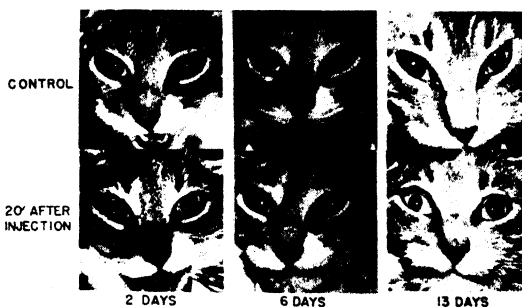


Fig. 3. EFFECT OF INTRAVENOUSLY INJECTED PILOCARPINE (0.4 mg/kg. body weight) upon right and left pupils of cat 37 from which the ciliary ganglia had been removed at an interval of 48 days. Note the appearance of maximal sensitivity in the left pupil 7 days after denervation and its greater miotic response as compared with that shown by the right pupils, denervated 35 days previously.

vein on the following day. The pupillary response to 0.01 mg. of the drug given 1 to 6 days after operation was not maximal in 10 experiments on 6 cats. In every animal maximal sensitization was observed by the eighth day. The photographs (fig. 3) of a bilaterally ciliary ganglionectomized cat show clearly the greater miotic effect of pilocarpine on the denervated pupil 7 days after operation (lower right photograph) as compared with that observed on the second day (lower left photograph).

Factors Influencing the Sensitivity of the Denervated Pupil to Pilocarpine. When pilocarpine was administered by vein there was a marked decline in the response of the pupil after several doses. The first dose of the drug, whether given as early as 6 days or as late as 92 days after operation, always evoked an intense response; subsequent doses produced noticeably less constriction of the denervated pupil. Figure

2 shows that the response of the denervated pupil to a third dose of pilocarpine, given on the 13th day after operation, was considerably less than the response obtained on the 6th day. In 15 denervated sphincter preparations an attempt was made to analyze the observed 50 per cent reduction in responsiveness of the pupil to an optimum (0.4 mg/kg. body weight) dose of pilocarpine. The constriction of the denervated pupil in millimeters following the first injection of pilocarpine given 8 or more days after ciliary ganglionectomy was assumed to represent 100 per cent responsiveness. Table 1 shows both the time in days after operation at which the denervated sphincter was 50 per cent as responsive as after the first injection of the drug, and the total number of doses which had been given at this time. It is apparent that whereas a 50 per cent reduction in pupil responsiveness may occur at any time from 21 to 262 days after operation, such a reduction inevitably follows the administration of 4 to 6 doses of pilocarpine by vein. Thus, the correlation between the reduction in pupil responsiveness and the total number of doses administered is closer than that between pupil response and the number of elapsed days following denervation.

TABLE 1. TIME IN DAYS AFTER CILIARY GANGLIONECTOMY AND TOTAL NUMBER OF INTRAVENOUS INJECTIONS OF PILOCARPINE (0.4 MG/KG. BODY WEIGHT) GIVEN WHEN SENSITIVITY OF THE PUPIL HAD DECREASED TO 50 PER CENT

	CAT NO.														
	3R	3L	6R	6L	7R	13	14R	14L	17	12	20	23	25	32	33
Days after operation.	192	50	196	83	262	136	91	44	69	97	126	78	111	41	21
Total no. drug doses.	4	5	6	4	5	4	4	6	5	4	5	5	5	4	4

In 9 cats from which one ciliary ganglion had been removed, the opposite ganglion was extirpated at a time when at least 4 intravenous doses of pilocarpine had been given. Maximal sensitivity to pilocarpine was attained in the usual 5 to 8 days, while at the same time the responsiveness of the previously denervated pupil was markedly diminished. The more recently denervated sphincter showed the same reduction in response to repeated doses of the drug as had the opposite pupil. These experiments indicate that the repeated administration of pilocarpine to the pupil, prior to denervation, does not influence the pupil's response to the drug after removal of the ciliary ganglion. The effect of repeated injections of pilocarpine appears to be localized in the previously denervated and already sensitized sphincter.

In another series of 5 cats, bilateral ciliary ganglionectomy was performed in two stages, at intervals ranging from 20 to 59 days. When pilocarpine was administered by vein more than 8 days after the second ganglionectomy, both pupils constricted markedly in response to the first dose injected. However, the response of the more recently denervated pupil was always greater by one to 3 millimeters than was that of the chronically denervated pupil, indicating that the sensitivity to pilocarpine diminishes slightly but measurably with the passage of time. In these cats administration of 4 doses of pilocarpine, in a short period of time, resulted in a decrease in the responsiveness of both sphincters. Figure 4 demonstrates the similarity in responsiveness of 2 pupils denervated 20 days apart. The first dose of pilocarpine

evoked intense miosis in both pupils; the same dosage given repeatedly within 6 days resulted in markedly reduced responses indicating a rapid decrease in sensitization.

This reduction in sensitivity of the parasympathetically denervated pupil, following repeated injections of pilocarpine, was not demonstrable when the drug was given topically at either the highest or lowest levels of the dosages used (table 2). Moreover, the responsiveness of the denervated pupil to topically administered pilocarpine was not depressed by repeated injections of pilocarpine (fig. 5). Thus, the

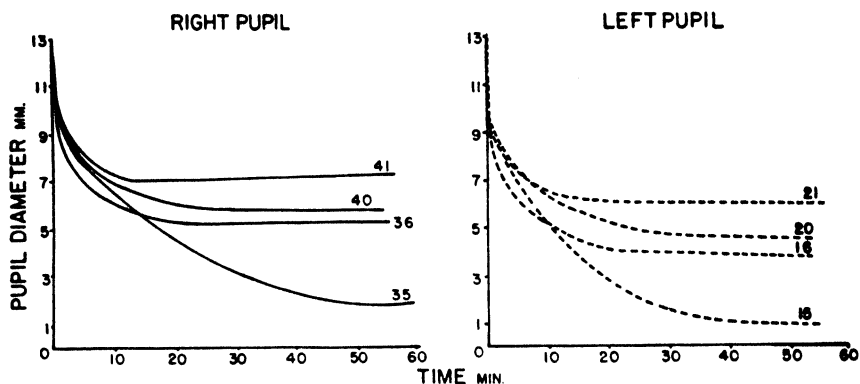


Fig. 4. EFFECT OF REPEATED INTRAVENOUS INJECTIONS OF PILOCARPINE (0.4 mg/kg. body weight) on the right and left pupils of cat 33. The pupils were denervated by excision of the ciliary ganglia at an interval of 20 days.

TABLE 2. MAXIMAL CONSTRICTION IN MILLIMETERS OF PARASYMPATHETICALLY DENERVATED PUPILS IN RESPONSE TO REPEATED LOCAL APPLICATION OF PILOCARPINE (0.01 MG/EYE).

DOSE NO.	CAT 23	CAT 34	CAT 35	CAT 36	CAT 37
1	11	10	11	10	11
2	10	9	9	11	10
3	11	11	9	9	8
4	10	11	10	9	9
5	10	8	9	9	10
6		9	10		10

phenomenon described above which resembles tolerance was observed only when the drug was given intravenously. However, several weeks after operation, the denervated pupil began to show decreasing miotic responses to topical pilocarpine. This decrease in sensitization was not related to the number of previous applications and reached a plateau after about 200 days (table 3).

DISCUSSION

The observations on the sensitization of the denervated pupil to pilocarpine found in the above experiments differ in two respects from those reported by Anderson (1). The development of maximal sensitization immediately after operation was not

observed in the present experiments, nor did the denervated pupil remain maximally sensitized. Indeed, the experiments indicate that there is a 5 to 8 days' latency between the time of denervation and the appearance of maximal sensitivity, as well as a progressive decline in sensitivity with time once maximal sensitization has been attained. The dose of pilocarpine employed by Anderson (1) was probably supra-maximal and, therefore, masked the finer gradations of the sensitization phenomenon. The truth of this statement is shown in the above experiments in which maximal

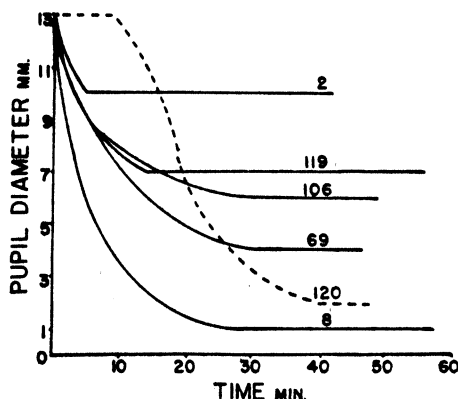


Fig. 5. EFFECT OF REPEATED INTRAVENOUS INJECTIONS OF PILOCARPINE (0.4 mg/kg. body weight) upon the right pupil of cat 17 after excision of the ciliary ganglion, as compared with the effect (dotted line) of locally applied pilocarpine (0.04 mg/eye).

TABLE 3. MAXIMAL CONSTRICTION IN MILLIMETERS OF PUPILS IN RESPONSE TO LOCALLY ADMINISTERED PILOCARPINE (0.04 AND 0.01 MG/EYE) AT VARIOUS TIMES AFTER REMOVAL OF CILIARY GANGLION (NOTE THE PROGRESSIVE DECLINE IN RESPONSIVENESS WHICH REACHES A PLATEAU AFTER 200 DAYS)

TOPICAL—0.04MG/EYE			TOPICAL—0.01 MG/EYE	
Days after operation	No. exper.	Max. decrease pupil	No. exper.	Max. decrease pupil
		mm.		mm.
1-49	15	11	19	10
50-99	12	9	10	8
100-199	13	8	5	7
200-299	10	6	4	4
300-399	9	6	4	4

sensitization was found immediately after denervation when a dose amounting to only one-tenth that used by Anderson was employed.

The results obtained with intravenously administered pilocarpine are, with one exception, similar to those obtained when pilocarpine was given topically. Maximal sensitivity developed by the 5th to 8th day following operation, an observation which agrees well with the times reported by Keil and Root (3) who used intravenously administered acetylcholine. With the passage of time the sensitivity of the denervated pupil to pilocarpine decreases. Repeated intravenous injections of pilocarpine are associated with a decrease in the sensitivity of the pupil which does not occur when the drug is given topically. The development of 'tolerance' to intravenously administered pilocarpine is difficult to explain. It does not seem likely that the decreased responsiveness to successive intravenous doses of pilocarpine can be caused by stor-

age, or inadequate destruction of pilocarpine, for the same decrease is seen when the drug is given at sufficiently widely spaced intervals to allow complete elimination from the body of the previous dose. Such a possibility is also refuted by the fact that almost daily doses of pilocarpine, instilled into the conjunctival sac, produce no analogous decline in sensitivity. It seems equally unlikely that systemically administered pilocarpine alone damages small, ocular blood vessels, causing a diminished blood flow to the sphincter, since pupils denervated after many doses of pilocarpine have been administered respond maximally on the first injection following denervation. For the same reason the suggestion that the injection of large quantities of pilocarpine into the blood stream results in the formation of a compound which effectively antagonizes the miotic effects of later injections of pilocarpine can be excluded.

Of all the theories which have been advanced to explain the sensitization phenomenon, the conception that denervation renders the cell abnormally permeable to the mediator substance as well as other agents (4) has received the greatest support. The gradual development of sensitization suggests that following denervation the cell or cell membrane undergoes some fundamental alteration in structure, probably chemical in nature. The gradual reduction in sensitization with time may be related to independent structural recovery of the cell or to regeneration of nerve fibers to the denervated cell (1, 5).

SUMMARY

Removal of the ciliary ganglion in the cat produces marked sensitization of the sphincter of the iris to pilocarpine, given either by vein (0.4 mg/kg. body weight) or instilled directly into the conjunctival sac (0.01 to 0.04 mg/eye). Sensitization of the pupil to threshold doses of pilocarpine, given systemically or topically, occurs immediately after removal of the ciliary ganglion, but is not maximal until 5 or 8 days following denervation. When supra-maximal amounts of pilocarpine (0.04 to 0.4 mg/eye) are administered topically, sensitization is maximal 24 hours after denervation. Intravenous injection of 4 to 6 doses of pilocarpine results in a decrease of 50 per cent or more in the sensitivity of the pupil. Repeated intravenous injection of pilocarpine does not alter the development of sensitization in a pupil which is subsequently parasympathetically denervated. Repeated local application of pilocarpine does not alter the sensitivity of the denervated pupil to the drug, nor is sensitivity to topically administered pilocarpine depressed by previous intravenous injections. There is a slight but gradual decline in the sensitivity of the denervated pupil to pilocarpine, given either systemically or locally, with the passage of time. The decrease is apparent as early as 35 days after denervation. Sensitivity reaches minimum levels at some time after the two hundredth day and then tends to remain constant.

The author thanks Dr. Walter S. Root and Dr. S. C. Wang for advice and criticism given during the course of this investigation.

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RESPONSE OF THE PARASYMPATHETICALLY DENERVATED CAT'S PUPIL TO ESERINE¹

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THE preceding paper (1) presents experiments which show that the sensitivity of the parasympathetically denervated cat's pupil to pilocarpine, after an initial increase of several days duration, diminishes with the passage of time. This observation can be explained by regeneration of the previously interrupted parasympathetic fibers. In this connection Anderson's observations (2) are of interest. He found that the miosis produced by eserine disappeared 7 to 9 days after extirpation of the ciliary ganglion and returned 3 to 8 weeks after the operation. Anderson believed that the loss of the pupillary response to eserine was related to degeneration of the parasympathetic nerve supply to the sphincter and that its reappearance could be attributed to regeneration of the fibers. These experiments have been cited as evidence for the statement that preganglionic fibers can replace postganglionic cholinergic fibers (3). The importance attributed to Anderson's findings has led the author to repeat and extend his experiments.

PROCEDURE

Seventeen cats were subjected to unilateral or bilateral ciliary ganglionectomy (1). At various times after operation, 2 drops of one per cent eserine salicylate (Mallinckrodt) were administered to the unanesthetized animals by instillation into the conjunctival sac of both eyes. The change in horizontal diameter of the pupils was measured by means of a millimeter ruler. In the unilaterally ganglionectomized preparations, the normal eye served as a control. When the eserine response of the ganglionectomized sphincter had returned and had become consistent on several trials, the oculomotor nerve was sectioned intracranially. The nerve was approached by trephining the parietal bone and reflecting the temporal lobe. In one animal the oculomotor nerve was sectioned without previous excision of the ciliary ganglion. Nine to 10 days later, eserine was instilled in the conjunctival sac and this was done repeatedly during a subsequent 65-day period, the changes in the horizontal diameter of the pupil being measured at each application of the drug.

RESULTS

The instillation of 2 drops of one per cent eserine salicylate into the conjunctival sac was often followed by salivation, lacrimation, muscular tremor and loss of equi-

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librium. The normal pupil showed an intense miosis which was maximal 45 to 60 minutes after instillation and was apparent for as long as 48 hours. The pupil which had been decentralized by intracranial section of the oculomotor nerve responded to eserine as intensely as the normal pupil.

In 17 cats from which the ciliary ganglia had been removed, the normal response to eserine disappeared 7 to 9 days after operation and returned 30 or more days later. The reappearance of the response to eserine was gradual and usually approached a maximum in about 60 days. This is illustrated in figure 1 in which the

Fig. 1. RESPONSES OF RIGHT DENERVATED PUPIL of cat 13 to locally administered eserine 141, 270 and 319 days after excision of the ciliary ganglion. The 310th day was also the 21st day after intracranial section of the oculomotor nerve. Note the partial loss of response in the lower photograph at the extreme right.

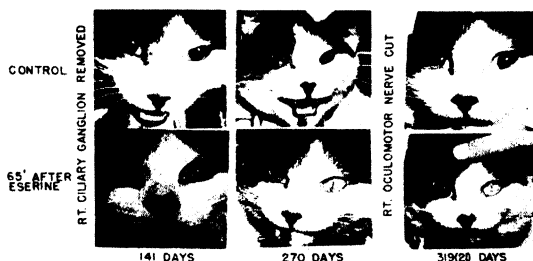


TABLE 1. RESPONSES OF 17 GANGLIONECTOMIZED PUPILS TO LOCALLY APPLIED ESERINE BEFORE AND AFTER INTRACRANIAL SECTION OF THE OCULOMOTOR NERVES

CAT NO.	DAYS AFTER CILIARY GANGLIONECTOMY	OCULOMOTOR NERVE	CONSTRUCTION OF PUPIL, MM.		CAT NO.	DAYS AFTER CILIARY GANGLIONECTOMY	OCULOMOTOR NERVE	CONSTRUCTION OF PUPIL, MM.	
			Before 3rd Nerve Cut	After 3rd Nerve Cut				Before 3rd Nerve Cut	After 3rd Nerve Cut
6 R	336	Seen and cut	8	4					
6 L	176	Seen and cut	10	4	10	140	Seen and cut	8	4
13	288	Seen and cut	7	4	17	206	Not found	10	10
32	89	Seen and cut	9	4	33	76	Not found	9	9
36	64	Seen and cut	11	1	3	393	Not found	8	
37	57	Seen and cut	10	1	7	371	Not found	9	
14	205	Seen and cut	9	5	12	210	Not found	9	
25	133	Seen and cut	8	0	27	50	Not found	8	
23	126	Seen and cut	10	6	20	138	Not found	8	

responses of the same pupil to 2 doses of eserine given at an interval of 120 days were essentially identical. The action of eserine upon a ganglionectomized but responsive sphincter generally produced an asymmetrical pupil (see lower left photograph, fig. 1). The irregularities in contour were seldom startling, but there was frequently puckering and flattening of either the malar or nasal portions of the iris.

The miotic effects of eserine on normal, ganglionectomized and decentralized pupils were abolished by the instillation of 2 drops of one per cent atropine sulphate into the conjunctival sac.

Intracranial sections of the oculomotor nerves were performed successfully in 10 of the 17 cats studied. A successful operation was considered to be one in which

the nerve was seen clearly and easily reflected off the middle fossa. In these circumstances section of the nerve was followed by the appearance of unmistakable ptosis on the operated side. The 7 unsuccessful operations in this series will be discussed later.

Tests with eserine, carried out 9 to 10 days after intracranial section of the oculomotor nerve, revealed in every instance partial loss of the response to eserine in the pupil on the operated side (table 1). In one animal the miotic response to eserine was lost completely (fig. 2); in the other 9 cats the pupillary constriction was reduced by at least 50 per cent (fig. 1). Thirty to 40 days after section of the oculomotor nerve, the response to eserine began to increase. By 60 days after the nerve section, all of the surviving animals showed a response to the drug in which the miosis approached that obtained before operation.

Ten or more days after intracranial section of the oculomotor nerve, the 10 preparations in which the operation had been performed successfully were tested for responsiveness to pilocarpine. The results obtained when pilocarpine (0.4 mg/kg. body weight) was administered intravenously indicated that no change in the sensitiv-

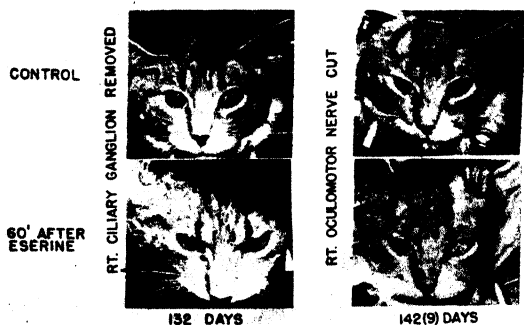


Fig. 2. RESPONSES OF RIGHT GANGLIONECTOMIZED PUPIL of cat 25 to locally applied eserine before and 9 days after intracranial section of the oculomotor nerve. Note the complete loss of the response to eserine in the lower right photograph.

ity of the pupil to the drug had occurred. The responsiveness of the ganglionectomized pupil to topically applied pilocarpine (0.01 mg/eye) increased in most instances after intracranial section of the oculomotor nerve. However, the increase was not marked, nor was it observed in every animal.

In 7 animals of this series, the oculomotor nerve was not seen when the temporal lobe was reflected. In every cat the operative field was explored extensively with the result that 5 cats died during the operation. Postmortem examination revealed no trace of the oculomotor nerve, although other structures were easily identified. Since in some animals the ciliary ganglion was removed with a large portion of the proximal root, the preganglionic fibers may have been present only as a stump and regeneration may have been hindered by the formation of scar tissue. In the 2 animals which survived the intracranial operation, but in which the oculomotor nerve was not seen, the miosis produced by eserine 10 days after operation was identical with that seen before operation. These experiments indicate that cortical manipulation alone does not cause any change in the response of the pupil to eserine. A summary of the results obtained on this group of 7 cats is included in table 1.

DISCUSSION

The above experiments confirm Anderson's (2) finding that the response to eserine lost a few days after ciliary ganglionectomy returns sometime later. We have assumed that continuity between the effector organ and the central nervous system has been re-established.

Since extensive manipulation of the brain alone does not alter the response of the previously ganglionectomized sphincter to eserine, the partial disappearance of this response, following intracranial section of the oculomotor nerve, must mean that preganglionic fibers can replace the postganglionic fibers which disappear after excision of the ciliary ganglion. The return of the eserine response in the ganglionectomized, decentralized pupil within 60 days after oculomotor nerve section is a further indication of regeneration of the preganglionic fibers.

However, regeneration of the preganglionic fibers to the ganglionectomized sphincter cannot be the only factor responsible for the recovery of the eserine response. If such were the case, the response to eserine should be abolished completely following oculomotor nerve section. The return of the eserine response to ganglionectomized pupils in which there is no evidence of the presence of oculomotor fibers casts additional doubt on the belief that the response is dependent solely upon an intact oculomotor nerve. According to Anderson (2) aberrant fibers from the 4th, 5th and 6th cranial nerves injured during the operation for removal of the ciliary ganglion may establish contact with the denervated sphincter. Since it is generally believed that any cholinergic fiber can functionally replace another cholinergic fiber (3), regeneration from other cranial nerves may have occurred in the 7 animals in which a response to eserine was observed in the absence of the oculomotor nerve. The accessory ciliary ganglia (2) may assume a functional role in the absence of the ciliary ganglion. Another possibility is that the denervated muscle of the sphincter may undergo some degree of independent recovery.

SUMMARY

Eserine was instilled in the conjunctival sacs of 17 cats in which unilateral or bilateral ciliary ganglionectomy had been performed. The miotic response to eserine which disappears 7 to 9 days after excision of the ciliary ganglion returns 30 to 60 days after the operation. After the reappearance of the response to eserine the oculomotor nerve was cut intracranially in 10 cats. There was a complete (1 cat) or partial (9 cats) loss of the eserine miosis 10 days after the operation. Sixty days after the cranial operation the magnitude of the response to eserine was of the same order as that present before section of the oculomotor nerve. In 7 cats in which ciliary ganglionectomy had been carried out, no trace of the oculomotor nerves could be found. The response to eserine which was present before the intracranial procedure was unchanged in the 2 animals which survived the cranial operation. The return of the pupillary response to eserine in cats from which the ciliary ganglion had been excised has been attributed partly to regeneration of preganglionic fibers and partly to other factors.

The author thanks Dr. S. C. Wang and Dr. Walter S. Root for advice and criticism given during the course of this investigation.

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MECHANISM OF THE ACUTE LETHAL EFFECT OF EPINEPHRINE IN RATS¹

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INJECTED epinephrine may cause two types of death: *a*) death from shock developing after prolonged, continuous administration and probably secondary to protracted vasoconstriction (1), and *b*) death occurring during the peak effect of a single injection. The latter has been variously attributed to many different factors (1-4), but its mechanism is incompletely understood in most species. Acute fatalities in rats following the administration of epinephrine, and also sudden myocardial failure in man, have been attributed to an accumulation of epinephrine or 'epinephrine-like' material (determined colorimetrically) in the myocardium (3, 5, 6).

A critical study of the lethal action of epinephrine in rats and mice is necessary in order to understand the mechanism of the marked protection provided by effective adrenergic blocking agents (4, 6-8), as well as to evaluate the specificity and validity of tests for adrenergic blocking potency which are based upon the protection afforded against the lethal effects of epinephrine (7, 8). Preliminary observations on acute epinephrine toxicity in rats and mice, incidental to studies on the protection afforded by Dibenamine, indicated that death was primarily due to respiratory rather than cardiac factors (4). The present experiments were designed to define more precisely the cause of acute death in rats following the administration of epinephrine.

METHODS

All experiments were performed on male Sprague-Dawley strain rats weighing over 300 grams. Pentobarbital sodium anesthesia (55 mg/kg. intraperitoneally) was employed. In certain controls, and in experiments involving carotid artery cannulation, heparin (800 U/kg.) was injected intravenously after induction of anesthesia. Toxic doses of epinephrine were administered intraperitoneally in the form of a 1:1000 commercial solution of epinephrine hydrochloride containing the usual preservatives (0.1% sodium bisulfite and 0.5% chlorotone). All doses are expressed in terms of the hydrochloride.

Mechanical stabilization of the arterial pressure was achieved by cannulating one carotid artery and attaching the cannula to a pressure bottle which provided an air buffer of about one liter. Pressure in the bottle was adjusted to the mean arterial pressure of each animal (usually 110 to 130 mm. Hg) prior to the injection of epinephrine. Intermittent positive-pressure artificial respiration with 100 per cent oxygen was administered through a tracheal cannula.

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Surviving animals were killed 45 minutes after the injection of epinephrine. Inasmuch as all fatalities in the control groups occurred much earlier (only one animal out of 23 died as long as 30 minutes after the injection of epinephrine), it appeared safe to assume that all animals alive at 45 minutes would survive.

Immediately after death the heart and lungs were removed, blotted dry and weighed. The heart was then ground with sand in a 10 per cent trichloroacetic acid solution. Epinephrine and epinephrine-like substances ('adsorbable chromogens') in the heart muscle were determined by the method of Shaw (8), as modified by Raab (3). Epinephrine solutions of known concentration were carried through the analytical procedure parallel with each group of tissue determinations and the tissue concentrations read against the standard in a photoelectric colorimeter in order to correct for any variations in procedure or reagents.

TABLE I. RESPONSE OF RATS TO EPINEPHRINE INJECTED INTRAPERITONEALLY

GROUP	TREATMENT	EPINEPHRINE mg/kg.	NO. ANIMALS	MORTALITY %	TIME OF DEATH min.	MYOCARDIAL 'EPINEPHRINE' μg/gm.	D.S.R.	LUNGS (% OF BODY WT.)
1	Pentobarbital; Heparin	0	7	0	Killed 30 min. after pentobarbital	0.94 ± .10	1.36 ± .04	0.59 ± .10
2	None	7	11	73	15 ± 2.0 ¹	2.34 ± .42 ¹	1.42 ± .11 ¹	0.66 ± .13 ¹
3	Pentobarbital; Heparin	7	12	02	19 ± 2.8 ¹	2.62 ± .25 ¹	1.71 ± .25 ¹	0.71 ± .08 ¹
4	Pentobarbital; Artificial respiration	7	10	0	Killed 45 min. after epinephrine	2.77 ± .34	1.49 ± .18	0.60 ± .08
5	Pentobarbital; Artificial respiration	25	8	0	Killed 45 min. after epinephrine	6.93 ± .43	2.01 ± .34	0.83 ± .10
6	Pentobarbital; Heparin; Pressure stabilization	7	9	0	Killed 45 min. after epinephrine	2.57 ± .16	1.55 ± .21	0.53 ± .06
7	Pentobarbital; Heparin; Pressure stabilization	15	4	100	19	3.03	1.78	0.67

¹ In animals dying spontaneously.

In order to correlate respiratory arrest and the subsequent resumption of spontaneous respiration with concurrent blood pressure changes, several rats were injected intraperitoneally with 15 mg/kg. of epinephrine and the arterial pressure was recorded by means of direct carotid cannulation and an Anderson glass capsule manometer. These experiments were carried out both with and without artificial respiration.

RESULTS

Three groups of control animals (1, 2 and 3 of table 1) provided a basis for evaluating the action of the various protective measures. Myocardial 'epinephrine' concentrations of animals injected with 7 mg/kg. of epinephrine with (group 3) or without (group 2) prior administration of pentobarbital and heparin were almost identical. All were significantly higher than the concentrations observed in animals not administered epinephrine (group 1). The interval between epinephrine injection

and death also was not significantly different in *groups 2* and *3*. Mortality was slightly higher in the group pretreated with pentobarbital and heparin, but the difference is not significant. The almost identical results, by all criteria, obtained with and without pretreatment with pentobarbital and heparin indicate that these agents do not significantly alter epinephrine toxicity in rats. This is in general agreement with reports of other workers. Raab (10) noted a slight prolongation of survival in rats under pentobarbital anesthesia, but no reduction in mortality, and Loew and Mice-tich (7) found that pentobarbital provided no protection against epinephrine toxicity in mice. The use of pentobarbital and heparin in experiments involving mechanical protection was therefore considered not to prejudice the results.

All of the control animals injected with epinephrine (*groups 2* and *3*) experienced respiratory difficulty prior to death. The respiration became irregular and gasping, and terminal hypoxic convulsions occurred in the unanesthetized animals. In anesthetized rats, shallow and irregular respiration was particularly evident, and was followed by weak convulsive moments. The thorax of each animal was opened immediately after the cessation of respiration and the heart was invariably found to be beating.

Rats provided with artificial respiration beginning shortly after the injection of epinephrine (*groups 4* and *5*) always survived well beyond the time of death of control animals even when 25 mg/kg. of epinephrine was injected (*group 5*). However, the myocardial 'epinephrine' concentration was equal to (*group 4*) or much greater than (*group 5*) that found in control animals at the time of death. These animals were invariably capable of maintaining adequate spontaneous respiration at the time of sacrifice (45 minutes after the injection of epinephrine).

All rats given 7 mg/kg. of epinephrine followed by mechanical stabilization of the arterial pressure (*group 6*) lived and exhibited no respiratory distress. In some experiments it was necessary to stop the exsanguination after blood amounting to about one per cent of the body weight had passed into the tube leading to the pressure bottle. After the administration of larger doses of epinephrine (15 mg/kg., *group 7*) the arterial pressure could not be stabilized until blood equal to about 3 per cent of the body weight had been lost. Although these animals maintained an adequate respiration, within 10 minutes the blood pressure began to drop very rapidly and they expired shortly thereafter.

The values for the D.S.R. (denominator of the specific ratio, comparing the colorimetric determinations made after acid and alkaline treatment of the tissue extracts, see ref. 3) were found to be quite variable. However, the higher values indicate that the chromogenic material (catechols) in the heart muscle contained a larger percentage of epinephrine after large doses of the agent had been administered.

Mean systemic arterial pressures recorded after the administration of 15 mg/kg. of epinephrine intraperitoneally followed a consistent pattern. The blood pressure began to rise within a minute after injection. The average increase in pressure was approximately 100 mm. Hg and about 75 per cent of the rise occurred before the end of the second minute. Respiration ceased 7 to 15 minutes after the injection; but the pressure continued to rise slightly after this, if adequate oxygenation was maintained by artificial respiration. By temporarily suspending artificial respiration at intervals

of 2 to 5 minutes it was found that spontaneous respiration again became adequate 10 to 25 minutes after it had stopped. In most cases spontaneous respiration was resumed while the blood pressure was within 10 mm. Hg of the level at which respiration had ceased.

DISCUSSION

The experiments reported above confirm our preliminary observation (4) that acute death from intraperitoneally injected epinephrine in rats is due to respiratory failure. It is apparent that changes in myocardial 'epinephrine' concentration are not causally related to death under these conditions. All animals in *groups 4 to 6* exhibited myocardial 'epinephrine' concentrations greater than 1.9 $\mu\text{g/gm.}$, which has been considered by Raab (3, 6) to be a uniformly lethal level. *Group 5* averaged 3.65 times the previously reported lethal concentration; yet the survival rate was 100 per cent when artificial respiration was provided. The normal rat myocardium, therefore, appears to be capable of functioning effectively in the presence of very high 'epinephrine' concentrations.

The use of artificial respiration to protect against epinephrine-induced respiratory embarrassment in rabbits was described many years ago by Auer and Gates (11). In this species (2, 12), as in the guinea pig (13), it is generally agreed that death from the administration of epinephrine is due primarily to pulmonary edema, and hence the mechanism of the protective action of positive-pressure artificial respiration is not difficult to visualize. However, our observations indicate that pulmonary edema is not an important feature of the lethal action of epinephrine in rats. Significant increases in respiratory tract fluid were not observed in any of the animals dying as a result of the epinephrine injections. The only group of animals showing a probably significant increase in lung weight was that in which the animals received 25 mg/kg. of epinephrine and were subsequently kept alive for 45 minutes with artificial respiration. As previously reported (4), massive pulmonary edema may finally develop in rats maintained on artificial respiration for several hours after the administration of very large doses of epinephrine. These results indicate that epinephrine may induce pulmonary edema in the rat, but that death from other causes ordinarily supervenes long before significant amounts of edema fluid have accumulated.

The precise mechanism by which epinephrine induces respiratory failure in rats is still not completely clear. The marked protection afforded by pressure stabilization in *group 6* indicates that the epinephrine-induced rise in blood pressure is an important etiological factor. This observation provides a basis for explaining the protection against epinephrine toxicity afforded by methacholine (7), and the fact that isopropylarterenol (N-isopropyl-norepinephrine) has a lower acute toxicity than epinephrine (14), although it has much more potent cardiac actions (15, 16).

It has long been known that epinephrine may produce both hyperpnea and apnea. Heymans and Bouckaert (17) reported that transient epinephrine apnea may be mediated reflexly through carotid and aortic baroreceptor mechanisms, although other investigators have noted epinephrine apnea after section of the afferent nerves subserving this function. Marri and Hauss (18) attribute epinephrine apnea in the

absence of the moderator nerves to changes in medullary circulation secondary to the increased arterial pressure, whereas Gernandt (19) has presented cogent evidence that a carotid chemoreceptor reflex is largely responsible. Previous observations reported from our laboratory indicate that epinephrine-induced apnea is not significantly altered when the blood pressure response is completely reversed by Dibenamine (20). Consequently, factors other than altered arterial pressure must be involved.

Available data do not provide clearcut evidence regarding the mechanism by which an epinephrine-induced rise in arterial pressure may cause respiratory failure. It seems probable that the mechanism involved in the slowly developing, fatal respiratory depression observed after the intraperitoneal injection of lethal doses of epinephrine is quite different from that involved in the immediate, transient apnea discussed above. Failure of Dibenamine to block the latter, although it is very effective in preventing the lethal effects of epinephrine, adds support to this conclusion.

It is not surprising that blood pressure stabilization failed to prevent the death of rats given 15 mg/kg. of epinephrine (*group 7*). In these animals the arterial pressure could not be maintained near preinjection levels without removing blood equal to 3 per cent or more of the total body weight. Sayers *et al.* (21) demonstrated that less than this amount removed over a period of one hour was lethal to 300-gram rats. They also observed that the shorter the bleeding time, the less hemorrhage was required to cause death. Inasmuch as the loss of blood in our experiments occurred within a few minutes it is reasonable to assume that the deaths in *group 7* were due to hemorrhagic shock. The antemortem hypotension is in agreement with this interpretation.

Experiments in which the mean arterial pressure was followed in rats given artificial respiration after the injection of lethal doses of epinephrine indicate that spontaneous respiration usually ceases shortly before the maximum pressure is reached and resumes while the pressure is still near the peak value. This suggests some type of respiratory accommodation to the effects of increased blood pressure, circulating epinephrine or both. Accommodation of the cyclopropane-sensitized myocardium to epinephrine has been reported (15), but the mechanism involved in the two instances may be quite different.

SUMMARY

Experiments designed to protect rats against lethal doses of epinephrine administered intraperitoneally have demonstrated the following points: 1) The lethal effect of epinephrine is not due to the accumulation of epinephrine or 'epinephrine-like' chromogens in the myocardium. 2) The animals may be protected against the lethal effect of epinephrine by either artificial respiration or systemic arterial pressure stabilization. 3) Pulmonary edema is not involved in the respiratory failure resulting from the administration of large doses of epinephrine.

It is concluded that the lethal effect of epinephrine in rats is dependent upon the induced rise in arterial pressure which in some way brings about respiratory arrest. This lethal effect appears to be unrelated to the transient 'epinephrine apnea' which is not dependent upon a rise in arterial pressure. On the basis of these observations

it must be concluded that the protection afforded against the lethal effect of epinephrine in rats, and presumably also in mice, by various drugs is not a measure of their specific adrenergic blocking activity.

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ADRENALINE APNEA IN THE MEDULLARY ANIMAL¹

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IN THE discovery of adrenaline Oliver and Sharpey-Schaefer noted a phenomenon that has since attracted attention out of proportion to its immediate importance because of inherent implications concerning the origin and regulation of the respiratory act (1). Upon intravenous injection of adrenaline, and during the period of elevated blood pressure and consequent reflex vagal slowing of the heart, respiration either diminishes or stops. Early accounts of this phenomenon generally invoked the vasoconstrictor action of the drug, attributing the apnea to a vasospastic anoxic paralysis of the respiratory center (2-5). This explanation lost favor when it was recognized that respiration alone was markedly influenced in this way when adrenaline was given to the decerebrate cat (6). Other functions mediated or regulated by neighboring regions of the brain stem such as decerebrate rigidity, limb reflexes, or the pupillary reflex, were less markedly altered, though changes might be expected if there existed a generalized brain stem anoxemia of a degree sufficient to paralyze respiration. Accordingly it was concluded that adrenaline acts directly and specifically on the medullary respiratory center (6).

Elucidation of the function of the carotid sinuses in the regulation of blood pressure led to the recognition that changes in blood pressure also influenced respiration. Injection of adrenaline into the trunk of an animal united to the head by its vagus nerves alone arrested respiratory movements in the head (7), and McDowall, noting the association of adrenaline apnea and vagal slowing of the heart, predicated a common mechanism for both effects (8). Isolating the carotid sinuses for perfusion independently of the systemic circulation, Heymans and Bouckaert were able to produce apnea and reflex fall in blood pressure by increasing the pressure of carotid sinus perfusion (9). It was concluded therefore that adrenaline apnea develops as part of the carotid sinus reflex, through reflex inhibition of the respiratory center. The existence of arrest of respiration via the carotid sinus reflex has been amply confirmed (10, 11), and some workers have claimed that this is the only action adrenaline exerts upon respiration (12).

There remains, nevertheless, evidence that prevents one from accepting reflex apnea as the sole factor in operation. Employing a compensator to minimize the elevation of blood pressure after administration of adrenaline, Roberts (4) and Kuno (13) nevertheless noted the appearance of apnea, which at times began before the rise in pressure. Brown observed apnea when the isolated head of an animal was per-

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fused with blood containing adrenaline (3). Nice and co-workers (14-17) found that the degree of apnea was neither proportional to the increase in blood pressure when moderate doses of adrenaline were used, nor was it as marked when the elevation in blood pressure was evoked by pituitrin instead of adrenaline. There are thus indications that, despite the arrest of respiration as a consequence of reflex action following administration of adrenaline, or in addition to it, adrenaline has a specific, direct, inhibitory action upon the respiratory center.

Recently it has been found (18, 19) that an animal deprived of all regions of the brain stem above the medulla still maintains a normal type of respiration which persists after section of the vagus and glossopharyngeal nerves. Such a preparation, representing the greatest simplification of the anatomical substratum of respiration yet achieved in the mammal, has afforded the opportunity to test the influence of adrenaline upon unmodified medullary respiration.

METHODS

In 16 dogs and 6 cats, out of a larger group in which isolated medullary breathing was established, the influence of intravenous administration of adrenaline was studied. In all animals the brain stem was sectioned in the rostral portion of the medulla just below the acoustic tubercles dorsally and the trapezoid bodies ventrally, usually after more rostral sections for other purposes. The vagi were sectioned in the neck. In all animals the carotids were ligated bilaterally below the carotid sinuses, which did not therefore participate in the generalized elevation of blood pressure evoked by the subsequent injections of adrenaline. In addition, in 5 dogs and 2 cats, the medulla was denervated by section of the glossopharyngeal and vagus nerves at their medullary origin. Results in both groups were identical.

In all experiments the respiratory pattern of the medullary preparation was permitted to stabilize for minutes to hours before adrenaline was injected, and it continued for equally significant intervals after injection. Respiration was recorded by tandem accordion pneumographs previously employed (18, 19).

RESULTS

After medullary transection in the vagotomized animal, respiration closely resembles that found after classical midcollicular decerebration with vagi intact in rate, regularity, depth and pattern. This is in marked contrast to respiration in midpontine preparations after vagotomy, which is apneustic in character, and in infra-pontine preparations in which it often has the character of Biot's periodic breathing. In general, the respiratory patterns are of 2 types, or combinations of the 2. One type is that described by Lumsden as 'gaspings', and by Hoff and Breckenridge as 'all-or-nothing,' a full, apparently maximum respiratory effort at relatively slow rates. Individual breaths in a series are usually fairly uniform in amplitude and regular in spacing; they show no signs of delay in the onset of expiration. Less regular in rhythm and in amplitude and of greater frequency, the other type gives the impression of ataxia, and may occur alone, or in combination with the all-or-nothing type. In figure 1, A and C illustrate how very closely indeed the respiration of the medullary preparation often resembles that of the same animal after the classical mid-collicular section with vagi intact.

In one dog, inexplicably, adrenaline injections were without effect. In all other animals, intravenous injection of 0.1 to 0.5 cc. of 1:1000 adrenaline produced com-

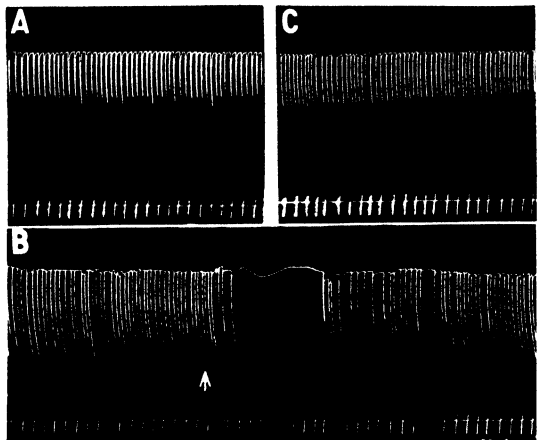
plete cessation of respiration for periods lasting from 30 seconds to 3 minutes (fig. 1*B*). Often a period of slowing followed that of arrest. Following resumption of respiration a second and third period of apnea returned in 2 cats, and in one other cat and in 2 dogs this phenomenon was accentuated to produce cycles of respiration and apnea resembling Biot's breathing (fig. 2) which continued for 3 to 20 cycles.

Following the apnea of adrenaline there frequently occurred an increment in the amplitude of respiration (fig. 3) which was especially marked in the rapid type of breathing. On 2 occasions this kind of breathing appeared for the first time following administration of adrenaline.

DISCUSSION

These experiments make it clear that respiration in the isolated medullary preparation, in which reflex inhibition via vagus or glossopharyngeal nerves is excluded, is nevertheless arrested by intravenous injection of adrenaline. It is logical to infer

Fig. 1. Dog *B* 40. July 21, 1949. Mid-collicular decerebration, carotids tied, 2:30 P.M. (A). Respiratory record, medullary section, bilateral vagotomy, 4:12 P.M. At 4:10 P.M., 0.4 cc. of 1:1000 adrenaline was given intravenously, producing apnea for one minute, 30 seconds. (B). Animal survived until 5:00 P.M. (C). Respiration at 4:30 P.M., demonstrating similarity of medullary respiration to control respiration in A. Time signals are 10 seconds, and inspiration is downward in this and subsequent figures.



therefore, in the light of these experiments and previous work, that adrenaline has a specific inhibitory influence upon the medullary respiratory center. There is nothing in the experiments reported here to arouse doubt of the reflex respiratory inhibitory effect of raised intracarotid pressure, and, of course, nothing to indicate any possible or probable biological function of the phenomenon. Nevertheless, they call attention to an interesting pharmacological property of the respiratory mechanism that cannot fail to have physiological importance.

Most closely the phenomenon approaches in its general characteristics the inhibition of sympathetic ganglionic transmission observed by Marrazzi after administration of adrenaline (20, 21). In their time courses, both of inhibition and of post-inhibitory rebound, the two effects of adrenaline are strikingly alike. Marrazzi has also described the reduction by adrenaline of the optical cortical potentials evoked by illumination of the eye, and of auditory cortical potentials in response to sound (22, 23), so that it appears that adrenaline arrest of respiration is part of a more

general phenomenon of adrenaline inhibition within the nervous system, the full extent and importance of which remains to be determined.

The inhibitory action of adrenaline at the sympathetic ganglionic synapse has suggested to Marrazzi "an adrenergic mechanism opposing and reciprocating with the cholinergic in the ganglia just as these two mechanisms are opposed at the neuro-effector junctions with very few exceptions" (21). The suggestion of Burn (24) that adrenaline sensitizes the tissue to acetylcholine has been rejected by Marrazzi as the explanation for adrenergic ganglionic inhibition on the grounds that during adrenaline inhibition, augmentation of the quantity of acetylcholine at the synapses by increase in preganglionic stimulation causes a break-through of impulses rather than further inhibition (21).

Whatever the final explanation of the phenomenon may be, the present experiments raise the question of hormonal participation in the initiation and maintenance

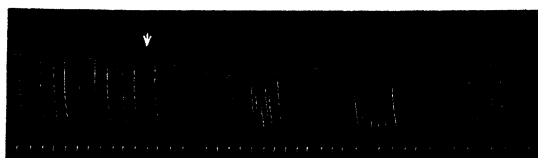


Fig. 2. *Cat S 44*. March 31, 1949. The animal was decerebellated, and brain stem was sectioned between the trapezoid bodies and the medulla. After respiration was stabilized for 40 minutes, the glossopharyngeal and vagal roots were

severed at their medullary origins. Fourteen minutes later 0.25 cc. of 1:1000 adrenaline was given intravenously producing apnea for 1 minute, 20 seconds. The apneic pauses were repeated, and breathing became cyclic. Biot's breathing persisted until death 30 minutes later.

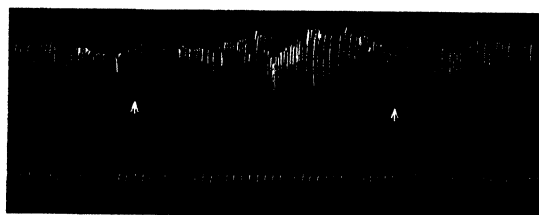


Fig. 3. *Dog B 30*. February 1, 1949. Mid-pontine decerebration at 11:10 A.M. Carotids tied. Vagi cut in neck at 11:25 A.M. and glossopharyngeals sectioned at medullary origin at 12:16 P.M. Medullary transection at 2:10 P.M. At 2:20 P.M. 2 successive injections of 0.5 cc. of 1:1000 adrenaline were given intravenously, producing short periods of apnea with well-marked post-inhibitory increase in respiratory amplitude.

of activity in the medullary respiratory center, and by analogy suggest a cholinergic excitatory mechanism reciprocating with an adrenergic inhibitory system. While cholinergic participation in respiration is one of the postulates of Gesell's electrotonic hypothesis, a reciprocal adrenergic system is rejected (25).

These results have significance in quite another direction. It has been postulated that the respiratory activity of the medullary preparation described here represents 'normal' respiration or the fundamental, periodic, respiratory activity which lies at the basis of normal breathing. Others have expressed quite different opinions. Lumsden (26, 27) has observed periodic respiratory activity in the medullary preparation but has described it as gasping in character and attributed to it no part in the genesis of normal respiration. Pitts, Magoun, and Ranson (28) have postulated that the basic activity of the medullary respiratory center is a tonic inspiratory drive without periodicity. In evaluating the contribution of the medulla to normal breathing, it is

therefore worth noting that the respiration of the medullary preparation responds to adrenaline in the same manner as in the intact animal or the decerebrate preparation. This supports the view that this respiration is essentially normal, and not a type of respiration unrelated to normal, or produced fortuitously by the stimulation of trauma, anoxia etc.

SUMMARY

Respiration in the vagotomized medullary animal, after carotid artery ligation or section of the glossopharyngeal nerves, is arrested by intravenous injections of adrenaline. It is concluded that apnea in these circumstances is due to direct inhibition of the respiratory center by adrenaline, akin to adrenaline inhibition of sympathetic ganglionic transmission and auditory and optical cortical potentials.

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ADRENERGIC AGENTS AND THE ADRENOCORTICOTROPHIC ACTIVITY OF THE ANTERIOR PITUITARY

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EPINEPHRINE has been shown to be effective in producing marked changes in cholesterol and ascorbic acid of the adrenal cortex (1). This effect does not occur in hypophysectomized animals. It is not clear, however, that epinephrine produces these changes by acting directly on the anterior pituitary (2, 3). Sayers (4) finds that injection of cortical steroids prevents this action of epinephrine and he suggests that epinephrine acts indirectly through its general metabolic effects. According to Sayers' concept the level of cortical hormones in the blood would control pituitary activity, and epinephrine would act by increasing the rate of utilization of these hormones.

The following papers recorded correlations of adrenal cortical activity with evidence of epinephrine release.

In the present study it was found that the quantity and duration of activity of exogenous epinephrine required to lower the adrenal ascorbic acid exceeded that required to increase the blood glucose. Tentatively accepting the probable identity of nor-epinephrine with sympathin E, it was employed to test the part played by adrenergic substances, other than epinephrine. The effect of nor-epinephrine on adrenal ascorbic acid as on blood glucose is about one-fourth that of epinephrine. Increase in blood glucose, therefore, could be used as evidence of sympathetic activity.

An attempt to dissociate the activity of the two components of the adrenal gland by adrenergic blocking agents, tetraethylammonium, Dibenamine, and ergotamine, was not successful and while elucidating some points did not give an unequivocal answer to the problem.

MATERIALS AND METHODS

Male Wistar rats weighing 80 to 180 gm. were maintained on Purina dog chow. In the absence of an air-conditioned room the rats were kept in a well ventilated laboratory hood. The temperature during winter months could easily be maintained at $26.5 \pm 2^\circ \text{C}$. During the late spring variations in room temperature caused some

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hypertrophy of the rats' adrenals, and high adrenal ascorbic acid. All animals were trained by daily handling at least 10 days before the experiment. The rats to be used for an experiment were placed in individual cages for 12 to 14 hours just preceding the experiment, food being excluded. Control animals were maintained under identical conditions.

After the experiment animals were killed by beheading and bled thoroughly before removal of the adrenals. This may account for the somewhat higher control values than reported by others. When the adrenals were removed without bleeding the ascorbic acid was about 10 per cent lower.

The adrenal ascorbic acid was determined by the method of Roe and Keuther (5) using the procedure of Sayers (6) for extraction. True sugar was determined by Nelson's (7) procedure using BaZn filtrate of Somogyi. When a series of bloods was taken 0.05 ml. was used, blood being collected from the tail. If only the terminal blood sugar was determined 0.1 cc. blood was used. The sensitivity of the method was increased by using a higher wave length than that recommended by Nelson.

Because of difficulties in obtaining consistent blood sugars many of the experiments were carried out under sodium pentobarbital (Nembutal) anesthesia. Barbiturates do not prevent epinephrine release (8) and epinephrine produces hyperglycemia in anesthetized rats (9). Although it has been stated by Ludewig and Chanutin (10) that Nembutal anesthesia per se lowers adrenal ascorbic acid in confirmation of Long (1), no effect on adrenal ascorbic acid was observed and the blood sugar tended to fall somewhat during the experiment.

The forms of stress used were heat, one hour at 38° C.; cold, one hour at 4° C.; hemorrhage, 2 per cent of body weight in 30 minutes; and insulin hypoglycemia and stimulation of the central end of sciatic nerve. All control animals were subjected to the same procedures of handling, injection of saline and dissection as the experimental animals. All animals were killed one hour after the start of the shocking procedure or 1½ hours after insulin injection.

All drugs were freshly prepared immediately before injection in isotonic saline. Epinephrine and nor-epinephrine solutions were protected with glutathione and were used within 5 minutes after solution. The amounts of various substances injected are expressed in units per 100-gram rat.

RESULTS

Effect of Epinephrine. It was important to find the level of exogenous epinephrine necessary to produce a lowering of the adrenal ascorbic acid and to find some independent measure of epinephrine activity to use in other forms of stress. The amount of epinephrine required to raise the blood sugar is small (9) and this effect was correlated with the lowering of the adrenal ascorbic acid.

The data in table 1 indicate that the hyperglycemic response is somewhat more sensitive to epinephrine than is the fall in adrenal ascorbic acid. The time over which epinephrine is active is important in the activation of the adrenal cortex. Four γ i.m. produced a slightly smaller effect than that noted by Long (1) with 0.02 mg/100 gm. injected subcutaneously. Sayers (11) using 4 γ has noted a marked fall in adrenal ascorbic acid. The maximum lowering of adrenal ascorbic acid appears to be produced

by intramuscular injection of 8 γ of epinephrine. This amounts to about 60 per cent of the total ascorbic acid within the gland.

Effect of Nor-Epinephrine. The consistency with which the action of nor-epinephrine can reproduce the effects of stimulation of sympathetic excitatory nerves has led many investigators to conclude that sympathin E and nor-epinephrine are identical (12-16). Since sympathetic stimulation in demedullated animals produces adrenergic effects, nor-epinephrine was used as the best available approach to the effects of sympathetic stimulation not involving the adrenal medulla. The pressor

TABLE 1. EFFECT OF EPINEPHRINE INJECTION ON ADRENAL ACID

	NO.	INJECTION		ADRENAL ASCORBIC	BLOOD GLUCOSE, mg/100 ml.			
		Route	Period		Time from start of injection, min.			
					Before	30-35	40-45	60-65
			min.	mg/100 gm.				
Controls Uninjected	12			514 ± 22				
Saline injected	6	i.v.	2	515 ± 11				
	12	i.v.	30	508 ± 17	68 ± 4	68 ± 6	62 ± 5	58 ± 5
	8	i.m.		515 ± 31				
Epinephrine, µg.								
0.5	5	i.v.	2	511 ± 18	68 ± 5	80 ± 4	72 ± 3	70 ± 7
1.0	7	i.v.	30	488 ± 20				
2.0	6	i.v.	2	495 ± 16	65 ± 3	90 ± 5	72 ± 5	68 ± 4
2.0	6	i.v.	30	382 ± 32	70 ± 2	95 ± 3	89 ± 4	78 ± 5
2.0	10	i.m.		405 ± 21	70 ± 5	85 ± 6	102 ± 6	82 ± 4
4.0	10	i.v.	30	308 ± 16				
4.0	5	i.m.		250 ± 23	65 ± 6	95 ± 4	110 ± 3	108 ± 7
4.0 ¹	6	i.m.		235 ± 18	78 ± 4	106 ± 7	118 ± 7	115 ± 7
8.0 ¹	5	i.m.		206 ± 10	80 ± 2	144 ± 6	135 ± 4	129 ± 6

Fourteen-hr.-fasted rats; i.v. injections in tail vein; 2-min. injections in volume of 0.2 cc. saline, 30-min. injections made in volume of 1 cc. saline injected at constant rate; i.m. injections in gluteal muscle 0.2 cc. saline. Rats killed 1 hr. after injection started. Nembutal anesthesia 5 mg/100-gm rat. i.p. in 0.2 cc. warm saline. ¹ Fed rats 4 hr. off food. Mean value and S.E.

activity of nor-epinephrine is about twice as great and the glycemic action about one-fourth that of epinephrine. According to Tainter (17) the acute toxicity of epinephrine is about three times that of nor-epinephrine.

The activity of various amounts of nor-epinephrine is given in table 2. The experiments were done during early summer at a time when rats' adrenals show some hypertrophy and high ascorbic acid values. The effect of 20 γ approximated that of 4 γ of epinephrine on both adrenal ascorbic acid and on blood sugar.

Experiments with Tetraethylammonium. This recently re-investigated drug has been convincingly shown by Acheson and Moe (18, 19) to paralyze autonomic ganglia. Since the adrenal medulla is essentially a sympathetic ganglion (20) it was

predicted that TEA would block epinephrine release in stress but still leave the animal susceptible to the direct action of exogenous epinephrine. The observations of Tepperman and Bogardus (21) appearing after these studies were completed showed that small doses of TEA (1 mg/100 gm.) produced no effect on the normal adrenal ascorbic acid nor did it prevent the fall due to injections of CCl₄. Preliminary experiments showed that much larger doses than this were required to prevent the reflex stimulation of the adrenals as measured by the increase in blood glucose after painful stimulation. Even amounts of 7.5 or 10.0 mg. doses were not sufficient to completely block the glycemic reaction.

It is apparent by inspection of table 3 that TEA of itself lowers the adrenal ascorbic acid. The drug is quite toxic in the large doses which were used in an effort to block reflex hyperglycemia. Five to 10 minutes following intra-muscular injection there was obvious respiratory difficulty, breathing was slow and deep with short periods of apnea resulting in marked cyanosis and some of the rats died of respiratory failure. The depressed state lasts 10 to 15 minutes, and 10 mg. was fatal to between one-third and one-half of the anesthetized rats. Inasmuch as anoxia is a well-known

TABLE 2. EFFECT OF NOR-EPINEPHRINE ON ADRENAL ASCORBIC AND BLOOD SUGAR

AMOUNT INJECTED	CONTROL ASCORBIC	NOR-EPINEPHRINE ASCORBIC	BLOOD GLUCOSE MAXIMAL INCREASE
$\mu\text{g.}$	mg./100 gm.	mg./100 gm.	mg./100 ml.
8 DL	(5) 561 ± 16	(5) 464 ± 31	0 ± 5
10 L	(7) 694 ± 20	(5) 424 ± 35	21 ± 5
20 L	(7) 694 ± 20	(5) 361 ± 17	33 ± 6
Epinephrine 4	(5) 694 ± 20	(5) 305 ± 21	42 ± 8

Nembutal anesthesia. Same controls used for 10 gamma, 20 gamma, and for epinephrine; control values high due to summer weather. Number in parentheses indicates animals used in each group. Mean value and S.E.

alarm stimulus (23-25) it was not surprising that there was lowering of adrenal ascorbic acid, but if the anoxia was acting through epinephrine release, one would expect hyperglycemia (26-28). However, in both control and drug treated animals, there was a significant downward trend in sugar values. The cortical activity in this case must have been independent of medullary activity.

Although not every rat responded by hyperglycemia to sciatic nerve stimulation, every rat did show a lowering of adrenal ascorbic acid, but there was no correlation between the ascorbic acid depletion and the blood sugar change. There is no doubt that maximum tolerated dose of TEA fails to prevent consistently the hyperglycemia of sensory nerve stimulation.

While either maximal tolerated doses of TEA or sensory stimulation alone produce a lowering of the adrenal ascorbic acid, there is no additive effect when the two stimuli are given to the same animal. This does not appear to be due to the fact that the response is already maximal since stimulation alone produced a greater fall in adrenal ascorbic acid than did either TEA or TEA + stimulation. TEA, therefore, does appear to block the further response to sensory nerve stimulation with regard to lowering the adrenal ascorbic acid but only in high concentrations.

Since TEA apparently blocks the changes in adrenal ascorbic acid caused by sensory nerve stimulation though not always preventing the occurrence of hyperglycemia, it was of importance to see if exogenous epinephrine would cause an additive reduction when given to TEA-treated animals. No further reduction on injection of 4 γ of epinephrine in TEA-treated animals was observed despite the fact that a maximal response is not caused by the TEA alone (table 3). Furthermore, while the blood sugar values show small increases, they are insignificant when compared with values obtained in normal animals.

Dibenamine Experiments. The most prominent action of Dibenamine (29) is a blocking of the pressor effects of epinephrine and sympathetic activity. This block

TABLE 3. EFFECT OF TEA ON BLOOD GLUCOSE AND ADRENAL ASCORBIC

PROCEDURE	NUMBER	BLOOD GLUCOSE CHANGES				ADRENAL ASCORBIC
		Decrease		Increase		
		No.	Av.	No.	Av.	
			mg/100 ml.		mg/100 ml.	mg/100 gm.
Saline treated						
control	11	9	-9	2	+11	453 \pm 35
stimulated	11	5	-10	6	+14	230 \pm 27
epinephrine 4 γ	13	0		13	+50	250 \pm 23
TEA						
7.5 mg/100 gm.	4					287 \pm 22
10.0	8	5	-12	3	+3	303 \pm 34
TEA treated and stimulated						
5.0 mg/100 gm.	8	3	-11	5	+13	241 \pm 23
7.5	13	2	-14	11	+17	256 \pm 17
10.0	9	4	-14	5	+13	314 \pm 31
TEA-treated 10.0 mg. epinephrine 4 γ	8	2	-8	6	+9	300 \pm 10

The changes in blood glucose are shown by the number of animals showing an increase or decrease. The central end of the sciatic nerve stimulated 5 minutes.

develops slowly and may last for several days after a single injection. Dibenamine affects neither the release of adrenergic compounds on sympathetic stimulation nor their rate of destruction. Since the blocking is due to the effect of Dibenamine on the receptor organ it was of interest to study its effect on the action of epinephrine and of various forms of stress with regard to adrenal cortical activity.

In the first series of rats, slow intra-venous injection of Dibenamine was made over a period of 30 minutes under Nembutal anesthesia. Even at this rate of injection many animals died. In those surviving a period of 2 hours was allowed for full blocking to take place (30). The animals were then subjected to hemorrhage. They showed vasodilation and were extremely easy to bleed.

The injection of Dibenamine caused a lowering of the adrenal ascorbic acid so

that the control values in these experiments are low. Table 4 shows that further lowering due to hemorrhage was slight. This may have been due to the fact that nearly maximal lowering had been achieved by the effect of the Dibenamine.

Since Nickerson (30) had shown that the adrenergic blocking effects of Dibenamine persisted, a second series of experiments was run on animals 16 hours after the intra-peritoneal injection of 2.5 mg. of the drug. Under these conditions, as in the experiments of Tepperman and Bogardus (21), adrenal ascorbic acid had returned to normal or somewhat above.

TABLE 4. DIBENAMINE AND THE RESPONSE TO STRESS

	DRUG TREATED		NORMAL CONTROLS	
	No.	Ascorbic Acid	No.	Ascorbic Acid
		mg/100 gm.		mg/100 gm.
<i>Group I</i>				
Controls.....	10	307 \pm 25	8	515 \pm 11
Hemorrhage.....	12	253 \pm 12	10	240 \pm 8
<i>Group II</i>				
Controls.....	14	576 \pm 17	8	515 \pm 11
Epinephrine, 4 μ g.....	5	450 \pm 16	10	250 \pm 12
Epinephrine, 8 μ g.....	5	309 \pm 23	6	206 \pm 20
Insulin, 0.05 U.....	10	378 \pm 19	7	370 \pm 18
Insulin, 0.1 U.....	5	240 \pm 11	10	235 \pm 24

Group I, 0.1 mg. Dibenamine was injected i.v. after period of 2 hr. bleed to 2% of B.W. during 30 minutes. Animals killed 30 minutes later. *Group II*, Dibenamine 2.5 mg. i.p., 16 hr. before the experimental procedure. No. refers to number of rats in group. Mean value and S.E.

TABLE 5. EFFECT OF ERGOTAMINE ON ADRENAL ASCORBIC

	CONTROLS	COLD TREATED	EPINEPHRINE
Normal.....	(6) 481 \pm 12	(6) 256 \pm 12	(6) 206 \pm 10
Ergotamized.....	(11) 411 \pm 9	(6) 311 \pm 9	(8) 273 \pm 15

Adrenal ascorbic mg/100 gm.: mean and S.E. Cold-treated at 4° C. for 1 hr. Epinephrine injection 8 μ g/100-gm. rat. Number in parenthesis indicates animals in each group. Mean value and S.E.

Table 4 shows that 8 γ of epinephrine was required to produce a 50 per cent reduction in the adrenal ascorbic acid while in the normal animals 4 γ produce this effect. The response to insulin hypoglycemia was approximately normal. Insulin had its usual hypoglycemic action and the increase in blood glucose due to epinephrine approximated that observed in normal animals.

Effects of Ergotamine. Ergotamine like Dibenamine prevents pressor effects of sympathetic stimulation or of injected epinephrine. It blocks the contractile and secretory mechanism susceptible to adrenergic agents. Larger doses are required to inhibit the pressor response to splanchnic stimulation (31, 32). Unlike Dibenamine, ergotamine inhibits the glycemic response to epinephrine.

These experiments were conducted on unanesthetized rats since barbiturates have been shown to reduce adrenergic blocking by ergotamine (33). The rats were injected subcutaneously with 0.05 mg. ergotamine tartrate. After a period of one hour the animals were injected with epinephrine or subjected to stress of cold. Only minor changes occurred in the blood sugar level. The fall in adrenal ascorbic acid due to ergotamine alone was slight as compared with that due to Dibenamine or to TEA. While there was a marked decrease in ascorbic acid due to both cold and to epinephrine, it was somewhat less than that observed in the normal animal. That these animals suffered more from cold than the normal animals was apparent from the fall in body temperatures which amounted to around one degree as compared with the normals that maintained their temperature. There were also signs of visible shivering in these animals.

DISCUSSION

This investigation of the relative effects of epinephrine on blood glucose and on the adrenal ascorbic acid shows that the amount of epinephrine required to produce a decrease in the adrenal ascorbic acid was greater than that required to increase the blood glucose. This was also found to be true of nor-epinephrine. It was, therefore, possible to use changes in blood sugar as an index of epinephrine release accompanying various forms of stress.

The use of adrenal ascorbic acid as an index of cortical activity limits the use of the operative approach of denervation or demedullation because of the variable effects of these operations on the circulation on the adrenal cortex. Adrenergic blocking reagents were used in an attempt to dissociate the two components of the adrenal. Tetraethylammonium chloride (TEA) known to block autonomic ganglia, Dibenamine and ergotamine which block the excitatory effects of sympathetic stimulation and of epinephrine were tested. Unfortunately all of the drugs produced a lowering of the adrenal ascorbic acid. TEA produced the most severe effect and further lowering of adrenal ascorbic acid by the stress of painful stimulation or by epinephrine was not observed even though maximal lowering was not produced by the drug itself.

With Dibenamine the immediate effect of the drug was to lower the adrenal ascorbic acid. The adrenals recovered their normal ascorbic acid in a period of 16 hours. Since the adrenergic blocking lasts for several days tests made on the day following the injection of the drug showed that the adrenals responded to the stress of hemorrhage, of insulin hypoglycemia or to injection of epinephrine but to somewhat less degree than the normal animal. Blood glucose changes with both insulin and epinephrine were the same as in normal rats.

Ergotamine produced a small but significant drop in adrenal ascorbic acid and failed to prevent a further lowering when the animal was exposed to cold, insulin hypoglycemia or to epinephrine. As is well known, it did inhibit the glycemic response to epinephrine, but it had no effect on the hypoglycemic action of insulin.

It is clear from the experiments with nor-epinephrine that the effect of epinephrine on the anterior pituitary and adrenal cortex is not related to its pressor activity. That this effect is not related to the hyperglycemic action as suggested by Steeples and Jensen (34) is shown by comparing the action of epinephrine on ergotamized

animals, in which no hyperglycemia was produced, with Dibenamine-treated animals in which the hyperglycemic action was still evident. The lowering of the adrenal ascorbic acid occurred in both these groups. The fact that TEA produces such a marked lowering of the adrenal ascorbic, when it would be expected to prevent sympathetic stimulation, suggests that the adrenergic agents are not directly involved. But it is impossible to draw this conclusion because in TEA-treated animals, injected epinephrine produced such small changes in blood glucose that these determinations are not an adequate test of epinephrine release under these conditions.

SUMMARY

These experiments indicate that any effect produced on pituitary or adrenal cortex through epinephrine release are not mediated through the pressor effect nor through the hyperglycemic reaction. To produce a fall in adrenal ascorbic acid, injected epinephrine must act over a relatively long period and must be injected in quantities sufficient to increase the blood glucose. Nor-epinephrine produces a fall in adrenal ascorbic and an increase in blood sugar equal to that of epinephrine when four to five times the amount is used. This is in the reverse order of its pressor action. Adrenergic blocking reagents—TEA, Dibenamine and ergotamine—all lower the adrenal ascorbic acid when injected. With ergotamine and Dibenamine a further lowering is observed following epinephrine injection or after various forms of stress. No further decrease was noted after TEA.

None of the drugs used were capable of determining finally the importance of adrenergic agents in anterior pituitary and adrenal cortical activity.

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SODIUM PENTOBARBITAL ANESTHESIA AND THE RESPONSE OF THE ADRENAL CORTEX TO STRESS

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IN THIS study, sodium pentobarbital (Nembutal) has been employed to block epinephrine release in rats exposed to the stresses of cold, heat and hemorrhage. A search was made for an explanation of the observed variations in the amount of ascorbic acid disappearing from the adrenal glands of anesthetized rats exposed to cold. The variations appeared to be due to the differences in the depth of anesthesia produced in individual rats. Sayers (1) has also noted a failure of anesthetized rats to show a fall in adrenal ascorbic acid when exposed to cold. Unanesthetized rats consistently respond to a short period of cold stress (2).

In lightly anesthetized animals exposure to cold produced no hypothermia and as in normal animals the adrenal ascorbic was lowered. But in deeply anesthetized animals there was a marked hypothermia and no decrease in adrenal ascorbic. In the anesthetized rat the stresses of cold, heat or hemorrhage were not accompanied by the increase in blood glucose noted in unanesthetized animals. In spite of this evidence of lack of epinephrine release the adrenal ascorbic acid was lowered as in anesthetized animals by hyperthermia or hemorrhage.

METHODS

In the preceding paper it was shown that the amount of epinephrine or of nor-epinephrine required to produce a fall in adrenal ascorbic acid exceeded that required to produce an increase in blood glucose. Therefore increases in blood glucose were used in this investigation as evidence of epinephrine release sufficient to produce changes in adrenal ascorbic acid.

In experiments involving cold stress the rats were exposed in the relatively dry air of a cold box maintained at the desired temperature. They were in a wire cage in which they could move about freely. The animals subjected to heat were placed in an incubator well supplied with air, passed through water of the same temperature as the incubator. The temperature was so controlled as to produce a rise of 2° C. in the rectal temperature of the rat. To produce stress from hemorrhage, blood equal to 2 per cent of the body weight was withdrawn from the tail in a period of 40 minutes. Unanesthetized animals were placed in a sling during the procedure. Although they had previously been trained there was some struggling accompanying the bleeding.

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Carefully trained male white rats of the Sprague-Dawley strain weighing around 150 gm. were used. With 5 mg. Nembutal per 100-gm. rat, some animals proved to be more deeply anesthetized than others. When light anesthesia was desired 4 mg. was used. It was injected intraperitoneally in warmed saline. In all cases unanesthetized animals were given Nembutal 5 minutes before taking terminal blood sample from the tail, just before killing.

The temperatures were taken by inserting a thermometer through a slit made in the abdominal wall in anesthetized animals. This temperature in animals exposed to cold proved to be one to two degrees higher than the rectal temperature as observed with a small clinical thermometer placed as high in the rectum as possible. The groin temperature was somewhat lower than the rectal temperature.

Analytical procedures were the same as used in the previous paper (3).

RESULTS

Effects of Exposure to Cold. Two types of experiments were used: the effect of 20 minutes exposure to $-7 \pm 1^\circ \text{C.}$ and of one hour exposure to a temperature between $5 \pm 1^\circ \text{C.}$ All animals were killed at the end of one hour after the start of exposure. Either type of treatment had the same effect on the ascorbic acid content of the adrenals in unanesthetized, unrestrained animals and the results on 16 rats, 8 under each condition, are reported in table 1. These rats were able to nearly maintain their normal temperature, the blood sugar showed definite but variable increases, and the blood lactic was always elevated when the animals were removed from the cold box. At either temperature, the rats were active, running around the cage. Those at $+5^\circ \text{C.}$ showed no visible signs of shivering. In exposures to -7°C. , although the rats' internal temperature was nearly normal, their feet and legs were cold, and there was marked shivering when placed in the sling for drawing blood. Marked vasoconstriction was indicated by some difficulty in getting blood from the tail even after warming it. Long (2) found animals were able to maintain their temperature under these conditions.

Hamilton, Dresbach and Hamilton (4) find that the unanesthetized rat exposed to water bath temperatures 0.2° to 0.6° shows a rapid and marked fall in rectal temperature, the thermocouple being placed high in the rectum. Their animals were confined in a rubber glove to prevent wetting of the hair. They were immobilized and put in a vertical position in the water bath. The rectal temperature started to fall immediately and decreased in a linear fashion.

With inactive anesthetized animals, the results were variable, and the decrease in ascorbic was less in many of the rats than in the unanesthetized animals. In a fairly large proportion no decrease was observed. It was noted that some of the rats showed pilomotor reflexes and intense shivering and were inclined to curl into a ball, while others more deeply anesthetized were relaxed throughout the experiment and showed no pilomotor reaction and no shivering. Although all of the animals showed a fall in body temperature, it was less marked in the individuals that raised their heat production by shivering and showed pilomotor reflexes.

The rats were separated into two groups on the basis of these reactions. *Group A* contained all the rats showing shivering or pilomotor reflexes. As shown in table 1,

there was a marked decrease in adrenal ascorbic; the blood sugar showed only slight changes, more often a fall than an increase. The blood lactic acid was elevated as would be expected from the muscular activity.

In *Group B*, none of the animals showed visible signs of shivering. These animals remained relaxed and showed no pilomotor activity. All of the animals in which there

TABLE I. RESPONSE OF RATS ON EXPOSURE TO COLD

PROCEDURE	ADRENAL ASCORBIC	CHANGE IN BLOOD GLUCOSE	CHANGE IN BLOOD LACTIC	BODY TEMPERATURE	PROCEDURE	ADRENAL ASCORBIC	CHANGE IN BLOOD GLUCOSE	CHANGE IN BLOOD LACTIC	BODY TEMPERATURE
	mg/100 gm.	mg/100 ml.	mg/100 ml.	°C.		mg/100 gm.	mg/100 ml.	mg/100 ml.	°C.
Anesthetized Controls (10)	556 ± 16	-4 to -8	7.5 ± 2	38 ± .5	<i>Group B</i> Deep anesthesia	531 508	0 -5	5.9 8.3	33.1 30.0
Unanesthetized Controls (10)	480 ± 42	+6 to +12	12.6 ± 8	38 ± .5	-7 ± 1° C	495 535 478	-15 -3 -5	11.8 7.5 8.3	30.7 32.2 32.0
Unanesthetized 5 ± 1° C (8)	256 ± 12	+20 to +42	21 ± 6	37.5 ± .6		550 680	-10 -8	7.3 6.5	32.0 31.2
-7 ± 1° C (8)	240 ± 18	+27 to +52	30 ± 8	37.8 ± .5		309 361	-8 +2	18.2 19.0	36.3 36.4
<i>Group A</i> Light anesthesia -7 ± 1° C.	308 357 365 375 236 315 431 365 280 317	-2 -8 -5 -2 0 +10 -10 +5 +4 -2	11.4 15.2 21.0 16.5 19.4 18.7 20.5 25.7 19.2 23.0	36.7 37.5 36.9 36.8 36.9 36.2 35.4 36.5 36.0 36.5	5 ± 1° C	480 471 552 490 520	-7 -4 -8 -12 -7	9.5 6.5 7.6 6.6 7.3	34.0 32.2 34.0 33.1 32.2
5 ± 1° C	316 358 340 313 310	+2 0 -5 -4 -6	11.8 12.0 10.0 14.5 16.5	36.0 36.5 37.2 37.3 36.5					

Rats exposed 5 ± 1° C. for 1 hr. or -7 ± 1° C. for 20 min. at room temperature for 40 min. before killing. Number in parentheses refers to animals in group. Mean value and S.E.

was no decrease in adrenal ascorbic acid were in this group. Two of the 10 animals showed significant decreases in adrenal ascorbic and also a significant increase in blood lactic. The blood sugar in these rats was generally slightly lowered and the fall in body temperature was marked. A number of the more deeply anesthetized animals treated at -7° C. died shortly after they were removed from the cold box. These

animals also showed low blood sugars and normal ascorbic acid values. Their temperatures were markedly lowered to 20 or 25° C. They were cyanotic and showed high lactic acid due probably to cyanosis. It was also found in longer experiments that of the animals whose temperature dropped markedly, some failed to recover after the effect of the anesthetic wore off, even though they were warmed in an incubator to normal body temperature. These animals also showed a normal adrenal ascorbic acid. They were cyanotic, showed a low blood sugar and a high blood amino acid.

It is notable that the blood sugar levels in these two groups of animals are remarkably constant with no indication of epinephrine release as measured by this method. Pilomotor and vasomotor activity on the other hand would seem to indicate some sympathetic activity in *Group A*. If any epinephrine was released, the resulting output of glucose was no more than sufficient to correspond to the increased metabolism. Adrenal cortical activity in *Group A*, where the metabolic rate was increased by shivering and increased muscle tone without sign of elevated blood sugar,

TABLE 2. EFFECT OF HYPERTHERMIA

	NO.	ASCORBIC ACID mg/100 gm.	BLOOD GLUCOSE		BLOOD LACTIC		BODY TEMPERATURE °C.
			Initial	Final	Initial	Final	
			mg/100 ml.	mg/100 ml.	mg/100 ml.	mg/100 ml.	
Anesthetized							
Controls.....	10	556 ± 16	67 ± 3	63 ± 5	7.5 ± 2	7.6 ± 2	38 ± .5
Heat.....	12	280 ± 13	68 ± 2	72 ± 3	7.2 ± 2	7.4 ± 4	40.5 ± .4
Unanesthetized							
Controls.....	10	480 ± 42	71 ± 4	82 ± 5	9.2 ± 3	11.5 ± 4	38 ± .5
Heat.....	12	250 ± 14	68 ± 2	122 ± 3	7.2 ± 2	23.3 ± 2	41 ± .6

¹Anesthetized rats exposed to 38° C. saturated for 1 hr.; unanesthetized rats exposed to 37° C. saturated for 1 hr. Mean value and S.E.

suggests that elevation in metabolic rate may be the basis of increased cortical activity. While in the group that showed no shivering, the decrease in body temperature lowered the metabolic rate even below normal, and in these animals the ascorbic acid remained at normal levels.

Hyperthermia, produced by placing the animals in a heated atmosphere, always resulted in a lowering of the adrenal ascorbic if the body temperature was increased in agreement with the observation of Long (2). Vogt (5) found changes in adrenal cholesterol only in those animals that showed hyperthermia. The unanesthetized rats were extremely disturbed, and actively ran about the cage looking for an exit. There was a marked degree of salivation which they spread over their whole surface, making the fur wet. They placed themselves as near the air inlet as possible and when two were in the cage at once they were continually fighting for the favored position.

The activity of the animals within the cage must have substantially contributed to their increase in temperature. The incubator temperature required to raise the rectal temperature of normal rats 2° C. was about 37° C. when the box was nearly saturated with water vapor. With anesthetized animals the incubator had to be

maintained at 39° to produce a temperature of 41° C. No salivation occurred in these rats unless anesthesia was very light. An examination of the blood sugar values in table 2 shows a well-marked increase in the unanesthetized animals. In those under anesthesia a decrease in blood sugar was usually observed. All animals anesthetized or not that showed an increase of 2° or more in rectal temperature showed a fall in adrenal ascorbic acid. These experiments, therefore, dissociate the lowering of adrenal ascorbic acid and the release of epinephrine.

With hemorrhage as the form of stress applied, the amount of blood withdrawn was not enough to produce a state of shock in the animals. Two per cent of body weight was removed from the tail vein over a period of 40 minutes. This amount, as shown by Long and co-workers (2), is sufficient to produce a lowering of adrenal ascorbic acid but not enough to produce irreversible shock. This was confirmed in these experiments. All animals that were not killed for ascorbic acid determinations recovered, and, 24 hours following bleeding, there was an increase above the controls in both ascorbic acid and cholesterol of the adrenal gland.

TABLE 3. EFFECT OF HEMORRHAGE

	NO.	ADRENAL ASCORBIC mg/100 gm.	BLOOD GLUCOSE		BLOOD LACTIC	
			Initial mg/100 ml.	Final mg/100 ml.	Initial mg/100 ml.	Final mg/100 ml.
Anesthetized						
Controls.....	10	556 ± 16	67 ± 3	63 ± 5	7.5 ± 2	7.6 ± 2
Hemorrhage.....	9	285 ± 20	68 ± 3	60 ± 5	7.8 ± 2	38.0 ± 4
Unanesthetized						
Controls.....	10	480 ± 42	71 ± 4	82 ± 5	9.2 ± 3	11.5 ± 4
Hemorrhage.....	6	248 ± 30	72 ± 4	125 ± 8	8.6 ± 2	45.0 ± 5

Two % of body weight was withdrawn over a period of 40 minutes from tail. No. refers to number of animals in each group. Mean value and S.E.

In unanesthetized animals (table 3), there was an increase in blood sugar amounting to between 30 and 50 mg/100 ml. of blood. This was highest at the end of bleeding time and returned to normal in a period of one hour after the last sample of blood was taken. There was also some increase in blood lactic which may have been due to a certain amount of activity on the part of the unanesthetized animal. The plasma amino acid value was unaffected by the procedure and remained constant throughout the experiment.

In anesthetized animals, there was little change in the blood sugar. Wilhelmi and Long (6) have found increases followed by a decrease in blood sugar in anesthetized rats subjected to hemorrhage sufficient to produce shock. In early experiments, 14-hour-fasted rats were used, and later fed rats were used. Both types of rats responded under anesthesia to epinephrine with an increase in blood sugar so that the failure of these anesthetized animals to respond to hemorrhage by a hyperglycemia must have been due to a failure to liberate epinephrine rather than lack of liver glycogen.

There was a significant rise in blood lactic acid in the anesthetized animals and no change in amino acid nitrogen. The temperature of the animals had to be maintained artificially so that there was probably no increase in the metabolic rate.

The activation of the adrenal cortex due to hemorrhage in the anesthetized animal appeared to be independent of epinephrine release. The decrease in adrenal ascorbic was about the same with and without anesthesia so that the epinephrine effect in the unanesthetized animals appeared to add nothing to the reaction of the adrenal cortex to this mild type of hemorrhage.

DISCUSSION

While barbiturates have been shown to inhibit only slightly epinephrine release due to strong sensory stimulation in the cat (7), they do inhibit this release under the forms of stress used in these experiments. Penrod (8) has shown that in lightly anesthetized dogs there is a variable degree of increase in metabolic rate from exposure to cold by immersion in ice water. Some animals showed a marked increase while others showed only a moderate or no increase in oxygen consumption. He considers this an individual characteristic although he shows in one dog both types of response. When lightly anesthetized, the oxygen consumption increased markedly, but under deep anesthesia showed no increase, but a gradual fall as the body temperature decreased. Hall, Crismon and Chamberlin (9) also find that mechanisms against cold defense are inactive in anesthetized cats.

Nembutal anesthesia was shown (3) not to influence the action of exogenous epinephrine either on adrenal ascorbic acid or on blood sugar. In this series of experiments this anesthetic depressed the nervous system to the extent that with the forms of stress used there was little sympathetic stimulation. The obvious emotional disturbances accompanying the stressing procedures must have substantially contributed to sympathetic release observed in the unanesthetized animals.

The first line of defense of the body to these harmful procedures was thus removed. With both hemorrhage and heat this did not prevent the stimulation of the anterior pituitary with the resulting lowering of the adrenal ascorbic. With cold on the other hand when sympathetic activity was prevented the animals not too deeply anesthetized showed shivering, similar to Cannon's animals after total sympathectomy, and in these animals the adrenal ascorbic was lowered to about the same extent as in unanesthetized rats treated to the same exposure. Apparently the stimulus to the anterior pituitary in these anesthetized rats did not involve epinephrine release. In these animals the metabolic rate must have been increased to the extent that in spite of the increased loss of heat there was only a slight fall in body temperature. In two of these procedures, therefore, heating and chilling, increased metabolism is associated with reduced adrenal ascorbic acid; when deep anesthesia prevents the increase of muscular activity and thence of metabolism to the stimulus of cold, the ascorbic decrease also fails. In the third procedure hemorrhage, although no increase in metabolism was obvious, at least epinephrine increase was ruled out as the cause of the decrease in ascorbic acid, and this is true of the other two procedures.

The effect of mild hemorrhage on metabolic rate in rats is not known. More severe hemorrhage causes a decrease. From the tendency of the body temperature to

fall and the necessity of applying heat to keep it normal, it seems probable that there is not an over-all increase in metabolic rate in these animals. However the metabolism of the tissues is affected by this process, as shown by the elevated lactic acid in these experiments. This is presumably the result of partial anoxia, involving at least difficulty in maintaining the metabolic rate. The tendency for the body temperature to fall may have been due to the failure of other heat-regulating mechanisms. It would be of considerable interest to know the metabolic rate in such animals.

These experiments suggest that increased metabolism, or perhaps the need for such increase after hemorrhage, is an immediate stimulus in the activation of the anterior pituitary to release ACTH. They are consistent with the idea that the depletion of cortical hormone by the activity of body tissues is an adequate stimulus. Conditions of stress may induce adrenal cortical activity without evidence of epinephrine release as measured by blood sugar changes.

SUMMARY

Normal or lightly anesthetized animals exposed to cold $+5 \pm 1^\circ \text{C}$. for one hour or $-7 \pm 1^\circ \text{C}$. for 20 minutes show a decrease in adrenal ascorbic acid at the end of an hour. Although normal animals showed an increase in blood glucose, lightly anesthetized animals did not. Deeply anesthetized animals showed neither an increase in blood glucose nor a fall in adrenal ascorbic acid. With hemorrhage or heat as the form of stress, anesthetic sufficient to prevent the normally observed increase in blood glucose failed to influence the lowering of adrenal ascorbic. Apparently even light anesthesia is sufficient to inhibit epinephrine release due to this mild form of stimulation.

These results are incompatible with the hypothesis that epinephrine is essential to stimulation of release of adrenocorticotrophic hormone, and suggest that the elevated metabolic rate is important.

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EFFECT OF WORK UPON TOLERANCE OF THE NORMAL RAT FOR INTRAVENOUSLY ADMINISTERED GLUCOSE

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IT WAS shown by these experiments that the glucose tolerance of the anesthetized rat was increased above values for resting animals by at least 300 per cent during the stimulation of muscle.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of 200 ± 2 gm. Following a 24-hour fast they were anesthetized with phenobarbital sodium and were given continuous infusions of solutions of glucose with heparin for periods of 72 hours. The infusions were made into the jugular vein by means of a constant injection apparatus which delivered fluid from each of 12 syringes at the rate of 40 cc. per 24 hours. Heparin was given as an anticoagulant in the amount of 2 mg. per rat for the first 24 hours and 1.5 mg. per 24 hours per rat for the remaining 48 hours. The procedures used for the stimulation of muscle were according to Ingle (1) with the following modifications. A Nerve Stimulator, Model B, Upjohn, was used to stimulate muscle at the rate of 5 times per second. The duration of each pulse was 20 milliseconds and the intensity was 20 milliamperes. An electrode was placed on the lower tibia of the left hind leg and the second electrode on the contralateral back foot, thereby activating all of the musculature of both hind legs. The gastrocnemius muscle of the left hind leg was weighted with 100 gm. The distance that the weight was lifted was registered on automatic work recorders. Each recorder revolution represented approximately 400 gm. cm. of work. Temperature was constant at $28 \pm 0.5^{\circ}\text{C}$. Glucose was determined on tail blood at 6, 24, 48 and 72 hours by the method of Miller and Van Slyke (2).

EXPERIMENTS AND RESULTS

Twelve rats were tested in each group. A few of the animals were discarded because of hemorrhage or because of thrombosis of the jugular vein followed by leakage of the infusion fluid into the tissues. The numbers of animals upon which the averages are based and the data on glucose tolerance and work are given in table 1.

When rats were given infusions of 0.9 per cent saline without work the level of blood glucose (initial average of 96 mg. per cent) was well sustained. Glucose loads of 50, 75 and 100/100/hr. caused an initial rise in the level of blood glucose with a subsequent fall. A glucose load of 125/100/hr. caused a continual rise in the level of blood glucose and the development of glycosuria in all of the animals.

When rats were subjected to work without the administration of glucose the onset of hypoglycemia and 'fatigue' was rapid. All of the animals died before completion of the 72-hour period. Some of the rats were observed to convulse prior to death. Glucose loads of 100, 125, and 150/100/hr. were tolerated without causing hyperglycemia during any phase of the experiment. Glucose loads of 200, 225, 250, 275 and 300/100/hr. caused an initial hyperglycemia the level of which correlated with the size of the glucose load. During the last 24 hours of stimulation the blood glucose decreased in all of the animals. Some of the rats developed glycosuria during the first 48 hours but only one rat (glucose load of 300/100/hr.) excreted glucose during the final 24 hours of stimulation. The work output of the rats was greatly

TABLE 1. EFFECT OF WORK¹ UPON TOLERANCE OF NORMAL RAT FOR GLUCOSE GIVEN BY CONTINUOUS INTRAVENOUS INJECTION: AVERAGES

GLUCOSE LOAD	EXPERI- MENTAL CONDITION	NUMBER RATS	INITIAL RATE	6 HOURS		24 HOURS		48 HOURS		72 HOURS		TOTAL WORK
				Rate	Blood Glucose	Rate	Blood Glucose	Rate	Blood Glucose	Rate	Blood Glucose	
<i>Mg./ 100/hr.</i>					<i>Mg./%</i>		<i>Mg./%</i>		<i>Mg./%</i>		<i>Mg./%</i>	
0	work	12	40	18	25	8	32	4	36	0		33405
	no work	10			94		104		113		95	
50	no work	11			150		106		102		104	
75	no work	12			180		145		129		124	
100	work	10	33	39	111	20	110	17	106	18	115	93780
	no work	10			214		133		136		124	
125	work	10	46	35	120	28	104	28	107	22	113	113895
	no work	10			306		354		548		695	
150	work	10	37	40	115	20	98	15	117	15	115	94352
200	work	8	55	40	163	32	136	30	122	27	120	139194
225	work	8	46	39	181	31	242	28	146	20	139	127286
250	work	9	44	44	193	33	226	25	164	20	139	120716
275	work	10	55	51	219	36	181	29	168	37	90	152941
300	work	9	54	48	168	32	234	29	252	34	130	148328

¹ The values for work (rate and totals) are expressed as the number of recorder revolutions. Each recorder revolution represents approximately 400 gram-centimeters of work.

increased by the intravenous administration of glucose and was roughly correlated with the glucose load.

DISCUSSION

Although it is reasonable to postulate that the effect of work upon glucose tolerance represents an increase in the utilization of carbohydrate for energy purposes we have no information on the fate of the injected glucose. It was previously shown that the stimulation of muscle causes a marked increase in the glucose tolerance of the liverless rat (3).

Adaptation is operative under these experimental conditions for there was a striking increase in glucose tolerance during the latter third of the period of stimulation. The mechanisms of adaptation can be assumed to be extra-alimentary. We

have previously reported on the adaptation of normal rats to the forced-feeding of high carbohydrate diet (4).

It would be of interest to prolong the stimulation of muscle in parallel with intravenous feeding but extended studies are complicated by either thrombosis of the jugular vein when high concentrations of glucose are injected without heparin or by spontaneous hemorrhages into the gut and elsewhere when thrombi are prevented by the addition of heparin. Also it was not practical to test higher glucose loads since the high concentration of glucose causes an occasional 'freezing' of the plungers of the syringes. It may be possible to solve these difficulties by further technological improvements in our equipment.

The activity load which can be placed upon the rat by these methods is very great and it offers interesting possibilities for the study of the food factors required for optimal energy output over periods of several days.

SUMMARY

Male rats of 200 grams weight were fasted for 24 hours and were anesthetized with phenobarbital sodium and given continuous intravenous injections of solutions containing glucose with heparin for 72 hours. In one series of animals the tolerance for glucose was studied under resting conditions. In the second series of animals both hind legs were stimulated to contract 5 times per second during the entire experiment. The highest glucose load tolerated by the inactive rats was 100/100/hr. (100 mg. of glucose per 100 gm. of rat per hour). The stimulation of muscle caused a marked increase in the rate at which glucose was removed from the blood. Glucose loads of 100, 125 and 150/100/hr. were tolerated without causing hyperglycemia during any phase of the experiment. Glucose loads of 200, 225, 250, 275, and 300/100/hr. caused an initial hyperglycemia but during the last 24 hours of stimulation the blood glucose decreased to nearly normal values. The upper limit of glucose tolerance for the working rat was not reached in these experiments.

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STUDIES ON THE MECHANISM OF THE POTENTIATION OF CIRCULATORY EFFECTS OF HYPERTONIC SOLUTIONS RESULTING FROM ADMIXTURE OF THESE SOLUTIONS WITH HOMOLOGOUS BLOOD^{1,2}

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THE hypotension resulting from intravenous injection of mixtures of hypertonic NaCl solutions with homologous blood may differ markedly from the effects of administration of correspondingly concentrated solutions of the salt alone. The modification in response, which is seen in the anesthetized dog and rabbit but not in the cat, may be described as a potentiation with increased intensity and, in the case of the dog, a characteristic alteration in the time course of the fall in blood pressure (1). The experiments which will be described in the present communication were designed to analyze the mechanism of this phenomenon which appears to result from an interaction between blood and hypertonic NaCl. The findings indicate that a depressor substance is released from erythrocytes in these circumstances. Although the depressor substance has not been characterized completely, it resembles in its tested pharmacological properties adenosine and its derivatives.

METHODS

The first part of the experimental analysis was carried out on anesthetized dogs, rabbits and cats. The numbers of animals, their weight range and the type of anesthesia used are summarized in the first columns of table 1. Mean arterial pressure recordings were made from one of the large arteries (femoral or carotid) using a standard recording mercury manometer. In some instances, heart rates were estimated from the kymograph records. The effects upon the mean arterial pressure of hypertonic solutions and mixtures of heparinized homologous blood with 20 per cent NaCl were compared with the results following administration of various fluids derived from blood-20 per cent NaCl mixtures. Details of the preparation of the latter derived fluids will be described below. Control solutions (0.9 per cent NaCl or blood mixed with 0.9 per cent NaCl) were injected to assess the possible participation of mechanical and other effects of the rapid intravenous injections in the phenomena observed. As noted previously (1), such injections were never followed by significant alterations in mean arterial pressure or heart rate.

In addition, experiments were carried out to study the response of a standard smooth muscle preparation to administration of hypertonic solutions, mixtures of

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blood with 20 per cent NaCl and related fluids. Strips of guinea pig intestine (duodenum or ileum) were obtained from non-fasted animals sacrificed by concussion. The intestine was transferred as rapidly as possible to Tyrode's solution³ at 38°C. and subsequently suitable strips were prepared and suspended in a continuously oxygenated bath containing 5 ml. of Tyrode's solution maintained at 38°C. The spontaneous contractions of the strips were recorded isotonicly on a kymograph drum by means of a light, nearly balanced lever. Test fluids were injected into the bath through a small bore polythene tube attached at one end to a hypodermic needle and having its other end affixed along the length of the muscle support within the bath. This tube was perforated with small holes in about 20 places. Fluids injected

TABLE 1. MAXIMAL DECREASE IN MEAN ARTERIAL PRESSURE FOLLOWING RAPID INTRAVENOUS INJECTION OF HYPERTONIC NaCl, BLOOD-20% NaCl MIXTURES, AND RELATED FLUIDS

SPECIES (WT. RANGE, KGM.)	NO. OF EXPER.	MAXIMUM FALL IN MEAN ARTERIAL PRESSURE IN MM. Hg FOLLOWING INTRAVENOUS INJECTION OF						COMMENTS
		0.9% NaCl 20% NaCl mixtures	Blood-20% NaCl mixtures	SI ¹	CI ¹	SII ¹	CII ¹	
Dog (7.1- 14.7)	5	8-44 (24) ² (di- phasic)	36-72 (55) (mono- phasic)	18-46 (23) (di- phasic)	18-30 (26) (mono- phasic)	10-40 (30) (mono- phasic)	0-10 (5) (mono- phasic)	
Rabbit (1.5- 2.4)	7	13-17 (16) ²	35-69 (46)	18-43 (26)	28-40 (34)	36-50 (43)	32-46 (39)	Atropine sulfate (2 mg/kg.) given in 5 ex- per.
Cat (2.0- 3.7)	3	46-90 (69) ² 35-49 (40) ²	46-84 (61) 26-46 (37)	46-77 (64) 18-60 (39)	25-38 (33)	30 ³ 20 ³		Before vagot- omy After vagotomy

¹ See text for composition of the derived fluids SI, CI, SII, and CII (p. 511). ² Figures in parentheses represent average values. ³ Single value only.

into the tube through the hypodermic needle entered the bath in fine streams at points along the entire length of the muscle support, and consequently mixing of test fluids added in 0.5 ml. volumes was effected rapidly and without mechanical stimulation of the muscle. In most of the experiments, pieces of intestine 1.0 to 1.5 cm. in length were suspended in the bath in such a way that the activity of the longitudinal muscle fibers was recorded. In 4 additional experiments, sections of duodenum about 0.3 to 0.5 cm. in length were arranged for recording predominantly the circular muscle contractions. These latter tests were carried out because it has been noted that, in the case of the guinea pig small intestine (jejunum) in particular, strong contractions of the circular muscle may stimulate inhibition of the longitudinal

³ The solute composition of the Tyrode's solution, in mm/l., was as follows: Na⁺, 149.9; K⁺, 2.7; Ca⁺⁺, 1.8; Mg⁺⁺, 1.0; Cl⁻, 149.1; HCO₃⁻, 11.9; glucose, 5.5; measured pH, 7.97 ± 0.18.

fibers by causing lengthening of the smooth muscle strip in the conventional Magnus recording (2). The various fluids which were tested for their effect on the smooth muscle will be described in connection with their demonstrated effects. A total of 66 intestinal strips, obtained from 21 animals, were used. Where more than one test was carried out on a single strip, the Tyrode's solution of the bath was usually changed between tests.

Measurements were made of the chloride ion concentration of some of the test fluids derived from blood, both in the circulatory experiments and in the observations on smooth muscle of guinea pig small intestine. The mercurimetric method of Schales and Schales (3) was used, and in certain circumstances it was felt that such chloride analyses could be used as an index of the degree of hypertonicity of fluids derived from mixtures of blood with relatively large quantities of 20 per cent NaCl. Measurements of pH were carried out in some experiments by means of a Beckman portable pH meter.⁴

Preparation of Fluids Derived from Blood-20 Per cent NaCl Mixtures. Mixtures of heparinized blood with 20 per cent NaCl in ratios of 4 to 1 (dogs and cats) or 2 to 1 (rabbits) were prepared and separated by centrifugation into the plasma-containing supernatant fluid and the mass of formed elements. The latter were resuspended in 0.9 per cent NaCl to the original volume of the blood-20 per cent NaCl mixture. To avoid repetition of the description of the two fluids thus derived from blood-20 per cent NaCl mixtures, they will be designated as follows: SI, the plasma-containing supernatant fluid, and CI, the cells (all formed elements) resuspended in 0.9 per cent NaCl. Other fluids were obtained from the CI fraction by centrifugation. The resultant supernatant fluid will be designated as SII, and the residual cells resuspended in 0.9 per cent NaCl will be termed CII in the discussion below.

RESULTS

Circulatory Responses in Dogs, Rabbits and Cats. The effects of intravenous administration of these fluids into dogs and rabbits are summarized in table 1. In the dog, injection of fluid SI produced a fall in blood pressure which was diphasic in character and usually of moderate degree, whereas CI caused a typically monophasic depressor response. Injection of SI in the rabbit was followed by a fall in arterial pressure which ranged, in most instances from 18 to 22 mm. Hg (in one experiment, 43 mm. Hg), and the hypotension resulting from administration of CI was always greater than that caused by SI. In both species, injection of the plasma fraction SI caused depressor responses similar to the effects of equally concentrated solutions of NaCl alone, whereas the characteristic modifications of the hypotension associated with interaction between blood and the hypertonic agent appeared to be related to the presence of the formed elements in the injection fluid.

Results substantially identical with those outlined above were obtained in experiments on dogs in which blood was separated by centrifugation into plasma and cells prior to admixture with 20 per cent NaCl. Plasma mixed with hypertonic NaCl caused

⁴We thank Drs. Francis Ryan and John Gregg, of the Columbia University Zoology Department, and Dr. Edward King, of the Barnard College Department of Chemistry, for making available to us instruments utilized in making these analyses.

hypotension which was indistinguishable, both in character and intensity, from the effects of administration of correspondingly concentrated mixtures of 0.9 per cent NaCl with 20 per cent NaCl. Injection of the formed elements, whether washed or unwashed, when resuspended in isotonic saline and subsequently treated with 20 per cent NaCl resulted in hypotension similar to the responses following administration of whole blood with 20 per cent NaCl. In 3 experiments, heparinized blood was centrifuged and the buffy coat was freed mechanically from the packed red cells. The erythrocytes were then resuspended in 0.9 per cent NaCl, mixed with 20 per cent NaCl, and injected intravenously. Monophasic depressor responses followed such injections, just as when the entire mass of formed elements was used. The buffy coat fraction, suspended in isotonic saline and mixed with 20 per cent NaCl, usually caused a diphasic fall in pressure, although in one case a monophasic fall was observed. The buffy coat fraction was always somewhat contaminated with erythrocytes.

The findings described above indicate that interaction of 20 per cent NaCl with red blood cells, rather than with other blood components, accounts for the potentiation of hypotension caused by mixing homologous blood with hypertonic solutions. Of a number of possible hypotheses as to the nature of this interaction, only two seemed to be sufficiently probable to warrant experimental analysis: *a*) the physical properties of the red cells may be so altered, e.g. crenation, 'sludging' (4), as to affect their passage through the vascular system; *b*) following treatment with hypertonic solutions, red blood cells may release a depressor substance acting directly on circulatory structures simultaneously with the hypertonic agent itself. It must be noted that there is no evidence for the release of a substance such as postulated in (*b*) upon direct exposure of erythrocytes to hypertonic NaCl, for if such occurred, the substance should be detectable in the plasma fraction SI. It might be inferred, therefore, that this release of a depressor substance does not take place until readmission of the red cells into the circulatory stream, i.e. into normal plasma. In an attempt to test the two hypotheses outlined above, resuspended formed elements (CI) were centrifuged and thus separated into a supernatant fraction (SII), and a residual mass of cells which were resuspended in 0.9 per cent NaCl (CII). The supernatant fluids SII were in all cases moderately too deeply hemolyzed. They varied in *pH* from 7.13 to 7.28 (experiments on rabbits) and were somewhat hypertonic, for the chloride concentrations of these fluids ranged from 324 to 380 mEq/l. in tests on dogs and from 298 to 338 mEq/l. in the studies made on rabbits. On intravenous injection of SII, monophasic depressor responses varying from 10 to 40 mm. Hg were noted in dogs, whereas in rabbits more marked hypotension was observed (table 1). These hypotensive responses could not be accounted for by the moderate hypertonicity of SII, for in 5 experiments on rabbits the intravenous injection of 345 mEq/l. (2% NaCl), a solution approximating in chloride content the supernatant fluids SII, caused only brief falls in blood pressure which ranged from a maximum of 5 to 15 mm. Hg. The results following injection of the residual formed elements resuspended in 0.9 per cent NaCl, CII, were somewhat different in dogs as compared with the findings in rabbits, as shown in table 1. In the former species, administration of CII had negligible effects on the mean arterial pressure, but in rabbits the pressure was always lowered by such injections. The chloride ion content of the fluids CII was found to range from 176 to

222 mEq/l. in experiments on rabbits. Thus these fluids were only moderately hypertonic.

Of the two hypotheses listed above as possible mechanisms for the interaction of blood and hypertonic NaCl, only the second one is consistent with the findings that SII, a fluid entirely free of cellular elements, is markedly hypotensive in the rabbit and causes moderate monophasic depressor responses in the dog. It was inferred from the data, therefore, that dog and rabbit blood cells release a depressor substance following exposure to strongly hypertonic NaCl and subsequent return to an isotonic medium either *in vivo* (circulating plasma) or *in vitro* (0.9% NaCl used in preparation of CI, SII, etc.). In addition, some inferences could be drawn as to the nature of this postulated depressor substance. It could not be potassium ion, at least in the case of the dog, for in this species potassium ion exists at a relatively low concentration within the erythrocytes. In 4 experiments on rabbits under deep urethane-ether anesthesia blood-20 per cent NaCl mixtures were consistently noted to be far more hypotensive than equally concentrated solutions of NaCl alone. This finding rules out histamine as the hypothetical depressor agent since in rabbits anesthetized with ether or urethane histamine is, in adequate doses, pressor rather than depressor in action (5, 6). Finally, acetylcholine was also eliminated, since striking hypotensive responses to injection of blood-20 per cent NaCl mixtures and of the supernatant fluids SII were observed in 5 experiments on rabbits which had received injections of atropine sulfate (2 mg/kg. subcutaneously or intramuscularly). None of the evidence cited is against the possibility that the depressor agent may be one of the adenine derivatives or pharmacologically similar substances which are released from mammalian red blood cells under a variety of experimental conditions (7, 9).

A few preliminary results were also obtained in experiments on 3 anesthetized cats. As shown in table 1, the injection of a supernatant fluid SI, prepared in a manner identical with the procedure used in the experiments on dogs, caused a profound fall in blood pressure which was comparable in degree to the fall following injections of equally concentrated solutions of NaCl alone, or of blood-20 per cent NaCl mixtures. During the response to injection of all three of these fluids, marked vagal slowing of the heart occurred frequently. When formed elements separated from blood-20 per cent NaCl mixtures were resuspended in 0.9 per cent NaCl (CI) and injected intravenously, a definite but relatively slighter hypotension was noted. In one experiment it was found that a supernatant fluid identical in its preparation to the fluids SII described previously caused a moderate fall in pressure which was of the same order of magnitude as the fall after administration of CI. These results confirm the previous finding that admixture of homologous blood with hypertonic NaCl does not potentiate its effect in the cat under sodium pentobarbital anesthesia (1). Although the hypotensive response to injection of the fluids CI and SII might be interpreted as evidence for the release of a depressor agent from red cells in the case of the cat as in the rabbit, it must be noted that these fluids were somewhat hypertonic and this may account for the fall in mean arterial pressure following their administration. Data on the effects of intravenous injection into cats of comparably concentrated solutions of NaCl alone are not available at the present time.

Studies on Guinea Pig Intestinal Smooth Muscle. If a pharmacologically active

substance having strongly depressor properties is released from dog and rabbit blood cells following exposure to hypertonic solutions, it should be possible to demonstrate its occurrence using a test system other than the circulation of dogs and rabbits. For this reason, isolated guinea pig small intestine was studied. The smooth muscle was exposed to mixtures of guinea pig blood with 20 per cent NaCl, hypertonic NaCl solutions and various other test fluids. The results of a total of 116 tests carried out on longitudinal strips of guinea pig duodenum and ileum are summarized in table 2. It may be seen that mixtures of blood and 20 per cent NaCl in a ratio of 3 to 2 inhibited the gut in 70 per cent of the tests, a decrease in tone and usually of the frequency of the spontaneous contractions being observed in 10 per cent of the tests, excitation was noted, and in the remaining cases there was no effect. Such blood-20 per cent NaCl mixtures were strongly hypertonic, having an NaCl concentration of approximately 1500 m Eq/l. (8 to 9%). Comparably concentrated solutions of NaCl invariably caused excitation which was characterized by a sharp rise in tone and was accompanied, in general, by a decrease in the amplitude of the spontaneous contractions. As in the case of the circulatory responses of the dog and rabbit, therefore, it may be concluded that blood-hypertonic NaCl mixtures are quite different in their action upon the guinea pig gut from comparably concentrated solutions of NaCl alone.

Mixtures of guinea pig blood and 20 per cent NaCl were separated by centrifugation into a supernatant fluid, SI, consisting of slightly hemolyzed plasma of high NaCl content (1380 to 1580 mEq/l.) and the mass of formed elements which were subsequently resuspended in 0.85 per cent NaCl or Tyrode's solution. The latter mixtures were further separated by centrifugation into a deeply hemolyzed fluid, SII, having an NaCl concentration of about 360 to 430 m Eq/l. by analysis and a residual mass of formed elements. Tests upon duodenal and ileal strips showed that the supernatant fluids SI and SII had opposite effects upon the gut, for SI, like correspondingly concentrated solutions of NaCl, invariably caused excitation whereas SII as consistently inhibited the intestinal smooth muscle (table 2).

Control tests, summarized in the last three rows of table 2, showed that the addition to the bath of Tyrode's solution had no effect on the intestine in 76 per cent of the tests, whereas only slight inhibition or excitation occurred in other cases. Approximately isotonic NaCl (146 mEq/l.) caused excitation of the gut in about half the observations. As a final control, 3 parts of blood were mixed with 2 parts of isotonic NaCl instead of 20 per cent NaCl and the mixture was carried through the steps leading to the formation of a supernatant fluid, designated SII c (controle), which corresponded with SII in its preparation but differed from it in that the blood had not been exposed to hypertonic NaCl during its preparation. This fluid was without effect on the intestinal strips in half the tests, and in most of the others caused inhibition of the gut.

The experiments carried out on circular muscle strips gave no evidence for either pronounced excitation or inhibition of this muscle by any of the test substances used in the conditions of the experiment.

The results described above suggest that at least one substance capable of inhibiting the longitudinal muscle of guinea pig small intestine is released from the

formed elements of the blood following admixture with hypertonic NaCl and subsequent resuspension of the formed elements in an approximately isotonic medium (Tyrode's solution or 0.85% NaCl). When the supernatant SII was maintained at 100°C. in a water bath for 15 minutes at a *pH* of 7.13 to 7.57, and the precipitated plasma proteins and hemoglobin subsequently separated out by centrifugation, the supernatant fluid thus obtained still caused inhibition of the gut in all of the 7 cases in which this test was carried out. In 5 experiments the boiled supernatant fluid freed of precipitated proteins was adjusted to *pH* 1 to 2 by the addition of 2.5 *N* HCl and this fluid was maintained at 100°C. for 15 minutes. It was then cooled, readjusted to

TABLE 2. QUALITATIVE SUMMARY OF RESPONSES OF SMOOTH MUSCLE OF GUINEA PIG SMALL INTESTINE TO VARIOUS TEST FLUIDS

SOLUTION TESTED	NO. OF EXPER.	NO. OF TESTS	PERCENTAGE OF TOTAL NUMBER OF TESTS RESULTING IN		
			Excitation	Inhibition	No Response
Hypertonic solutions—					
259 mEq/l. NaCl.....	4	5	100	0	0
860-3450 mEq/l. NaCl.....	5	6	100	0	0
Blood-20% NaCl mixtures.....	4	10	10	70	20
SI ¹	9	12	100	0	0
SII ¹ —Initial test.....	19	19	0	100	0
After ATP.....	6	10	10	60	30
After SII heated at <i>pH</i> 7-8.....	7	8	75	25	0
SII ¹ —Heated 15' at <i>pH</i> 7-8.....	7	7	0	100	0
Control solutions					
Tyrode's.....	16	25	12	12	76
146 mEq/l. NaCl.....	6	6	43	0	57
SIIC ¹	5	8	12	38	50

¹ See text for composition of fluids SI, SII and SIIC.

pH 8 with 2.5 *N* NaOH, and tested for its effect upon the small intestine. In 7 out of 8 tests, this solution caused a brief and usually slight inhibition of the gut, followed by excitation and finally by inhibition of longer duration. In the one case in which this action was not observed, the gut was simply inhibited. It was concluded from these data that the active material released from the red cells is not destroyed by heating for 15 minutes at a slightly alkaline reaction, but the characteristics of the material appear to be modified by an additional 15 minutes at 100°C. in an acid medium. An observation which was made repeatedly but which is unexplained was that after an intestinal strip had been treated with SII heated at *pH* 7 to 8, it frequently was excited, rather than inhibited by a subsequent test with unheated SII.

Finally, the effects upon the guinea pig intestine, under the conditions of the

experiment, of the administration of adenosine triphosphate⁵ and excess potassium ions were tested, since the results described by previous workers on the effects of these materials on intestinal smooth muscle are somewhat conflicting (10-14). Concentrations of 1.6 μ g. of ATP per ml. of bath inhibited the gut in 11 out of 15 tests, and was without effect in the remaining four. Curiously, a second equal dose of ATP caused inhibition in only 4 out of 8 trials, and larger doses (7, 8 to 15.6 μ g ml.), especially on repeated administration, caused excitation instead of inhibition in about half of the trials. It was noted, too, that when an intestinal strip had been treated repeatedly with ATP administration of SII often was without effect or caused excitation, rather than inhibition as it normally did. Tyrode's solution with added KCl (K ion concentration, 21 mM) inhibited the gut in all tests, but on repetition of the administration of K ion the effect frequently reversed so that excitation of the intestinal strips occurred.

From observations described above, some conclusions may be drawn as to the nature of the substance released by guinea pig blood cells after exposure to hypertonic NaCl. This substance cannot be acetylcholine or histamine, for these compounds excite, rather than inhibit, the guinea pig intestine. The fact that the material is not destroyed by 15 minutes at 100°C. in a solution of pH 7 to 8 eliminates the possibility that it might be the partially characterized depressor substance Kallikrein (15). Under the conditions of the experiment it behaved like adenine derivatives and excess potassium ion. Its activity appeared to be modified by boiling in acid solution, which would not be anticipated of potassium ion, but might be expected in the case of adenine nucleoside and nucleotides since these compounds lose their physiological activity on complete hydrolysis of the adenine-ribose bond (11 and others). Its effect is also modified in some instances by pre-treatment of the gut with ATP, as is the effect of ATP itself.

DISCUSSION

Solutions derived from mixtures of dog, rabbit or guinea pig blood and strongly hypertonic NaCl appear to contain one or more pharmacologically active substances not distinguishable, on the basis of the tests used, from adenosine and its derivatives. This is not surprising in view of previous demonstrations of the existence of adenine nucleotides in red blood cells (16, 17, and others). Adenosine, adenylic acid, and adenosine diphosphate and triphosphate have been known for a long time to be powerful depressor agents in mammals, causing, among other effects, peripheral vasodilatation, bradycardia and impaired cardiac conduction with heart block in some species, and, in the cat only, marked vagal slowing of the heart (10, 18). It is interesting that we have observed all of these effects following the administration of strongly hypertonic solutions. The presence of incompletely characterized depressor substances in dog and guinea pig blood following traumatization of the blood or specific hemolysis has been described (19, 20). In addition, various workers have presented evidence, for the release of adenosine, adenylic acid, and ATP from red blood cells of the cat,

⁵ The sodium salt of adenosine triphosphate, $\text{Na}_4(\text{ATP}) \cdot 3\text{H}_2\text{O}$, obtained from Rohm and Haas Co., was used. Amounts of ATP were calculated as 0.78 times the corresponding weights of the dry salt.

dog, rabbit, hare and beef during clotting and shaking of blood (7, 9) and hemolysis with distilled water (8). It may be noted that the question has been raised whether or not the substances described are indeed specified adenine derivatives, or other compounds closely simulating the former in their effects on various biological test systems (12).

The postulated mechanism of the potentiation of hypertonic salt hypotension which occurs in dogs and rabbits when blood is mixed with the hypertonic agent before injection may be summarized briefly as follows: When red blood cells are treated with strongly concentrated solutions and are then replaced in an isotonic medium by injection into the blood stream, a depressor substance related, at least in its tested effects, to adenosine and adenine nucleotides is released and acts upon circulatory structures simultaneously with the hypertonic agent itself. Presumably such interaction between the blood cells and hypertonic solutions may occur even when the latter are injected directly, for during a short period of time there must exist a more or less definite boundary between the injected mass of salt solution and the circulating blood, and here blood cells would be exposed briefly to a medium having a high solute concentration. On the other hand, it is doubtful whether as many cells could be exposed to a highly concentrated medium when strongly hypertonic fluid is injected intravenously as when blood is mixed *in vitro* with such a fluid. This would account for the lesser degree of hypotension resulting from administration of salt solutions unmixed with blood. Release of the depressor substance from erythrocytes may occur as a result of hemolysis. It has been shown that red blood cells exposed to a strongly concentrated medium and subsequently transferred to an isotonic or slightly hypertonic solution hemolyze rapidly (21). This phenomenon, which was called 'paradoxical hypotonic hemolysis' by Söderström, was attributed to the penetration of solute through the erythrocyte membrane during exposure to the hypertonic solutions. On return to an isosmotic medium, the cells behave as if the previously isotonic fluid is now markedly hypotonic to them. The derived fluids SII described above as having strongly depressor effects in both dogs and rabbits were always hemolyzed, and hemolysis occurs *in vivo* following the intravenous injection of hypertonic solutions. We have noted a progressively increasing intensity of coloration with hemoglobin in samples of blood removed from dogs, cats and rabbits following repeated injections of hypertonic NaCl. Hemoglobinuria was observed often toward the end of experiments of moderate to long duration. Similarly, hemoglobinemia was described by Robertson and Barrett (22) after relatively slow injections of 30 per cent NaCl in nembutalized cats.

Although presenting a general explanation for the potentiation of the circulatory effects of hypertonic solutions by admixture with homologous blood, the hypothesis outlined above fails to account in detail for the highly constant difference in the timing of the hypotensive responses seen in the dog following injection of blood-20 per cent NaCl (monophasic depression) as compared with saline-20 per cent NaCl mixtures (diphasic depression). Furthermore, at the present time it is not possible to explain the demonstrated variation between the cat, on the one hand, and the dog and rabbit on the other with respect to their reactions to hypertonic salt solutions and blood-hypertonic salt mixtures. Thus, as noted above, other in-

vestigators have described the release of adenosine-like substances from the erythrocytes of all three species. In addition, the red blood cells of the cat, like those of the dog, appear to be more susceptible to 'paradoxical hypotonic hemolysis' than those of the rabbit and guinea pig (23). It has been suggested, however, that the cat is somewhat less sensitive to adenosine than the rabbit, dog and monkey (18). At the moment, this seems to be the only available clue as to the nature of the difference between the three species.

SUMMARY

When the blood of dogs and rabbits is mixed with strongly concentrated solutions of NaCl, and the formed elements are subsequently resuspended in isotonic saline, a depressor substance is released from the cells. The substance has not been identified, but it is not potassium ion, acetylcholine or histamine. It is suggested that this material, and a smooth muscle-inhibiting substance liberated from guinea pig blood cells in the same circumstances, may be similar to, or identical with, the adenosine-like compounds released from mammalian erythrocytes under various experimental conditions (clotting or shaking of blood; hemolysis). The release of this depressor material is thought to account, at least in part, for the potentiation of the hypotensive effects of hypertonic solutions resulting from their admixture with homologous blood.

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OBSERVATIONS ON HYPOTENSION FOLLOWING INTRA- VENOUS INJECTION OF STRONGLY HYPERTONIC SOLUTIONS MIXED WITH HOMOLOGOUS BLOOD¹

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WHEN strongly hypertonic solutions of sodium chloride or glucose are administered by rapid intravenous injection to anesthetized dogs, the resulting brief hypotension tends to occur in two phases. The first of these sets in rapidly and is of moderate degree. After a plateau or transient rise in mean arterial pressure, a second phase is seen in which the fall in pressure is considerably more marked than in the first phase. In preliminary experiments we have noted, however, that the diphasic character of the response to such injections is altered to a simple monophasic fall in pressure if the hypertonic agent is mixed with homologous blood prior to injection. This finding appeared to throw some light on the mechanism of the hypertonic solution hypotension, and for this reason experiments were made to study the phenomenon in more detail. The results of these experiments, with supplementary observations on the character of the depressor effects of similar injections in rabbits and cats, are described below.

METHODS

Nine dogs, 6 cats and 4 rabbits were utilized in this series of experiments. The dogs and cats were anesthetized with sodium pentobarbital (36 mg. kg. intravenously or intraperitoneally) and the rabbits with sodium pentobarbital (50 mg. kg. intraperitoneally) supplemented with ether applied with a face mask. The animals varied in weight as follows: dogs, 7.4 to 17.4 kg.; cats, 2.0 to 3.7 kg.; rabbits, 1.9 to 2.4 kg. Circulatory changes following the rapid intravenous injection of hypertonic fluids were analyzed according to the methods described elsewhere (3, 4). In general, mean arterial pressures and heart rates were recorded with a mercury manometer, and supplementary data were obtained, where necessary, on systolic, diastolic and pulse pressures using a Hamilton metallic membrane manometer. Analyses were also made of plethysmographic records showing alterations in the volume of one of the hind feet. The method used, and some of the results from 3 experiments on dogs in which the measurements were made, have been described in a separate communication (4).

The hypertonic fluids injected included 20 per cent NaCl and mixtures of 20 per cent NaCl with homologous blood or with 0.9 per cent NaCl. The ratios of blood or 0.9 per cent NaCl to 20 per cent NaCl in these injection fluids varied somewhat in different experiments, but were, in general, 4 to 1 in the experiments on dogs and

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cats, and 2 to 1 in the experiments on rabbits. Thus the final concentration of NaCl in the injection mixtures was of the order of 4.7 to 7.3 per cent (0.8 to 1.3 M). In all experiments, the effects of the intravenous injection of mixtures of blood with 20 per cent NaCl were compared with the results following administration of equal volumes of solutions of equal effective concentration prepared by mixing 0.9 per cent NaCl with 20 per cent NaCl in the same relative proportions as blood and 20 per cent NaCl. For simplicity, these fluids will be referred to below as 'blood-20 per cent NaCl mixtures' and 'saline-20 per cent NaCl mixtures.' The blood which was utilized for preparing injection mixtures was withdrawn from the same animal into which the injection was to be made. If an anticoagulant was needed, heparin was used. It may be noted that the presence of heparin in the injection mixture was without any detectable effect upon the circulatory response. Usually mixtures of blood with hypertonic NaCl were administered within one to 2 minutes of preparation. In a few experiments a study was made of the effects of permitting blood-20 per cent NaCl mixtures to stand for 20 minutes or more before injection. All injections were

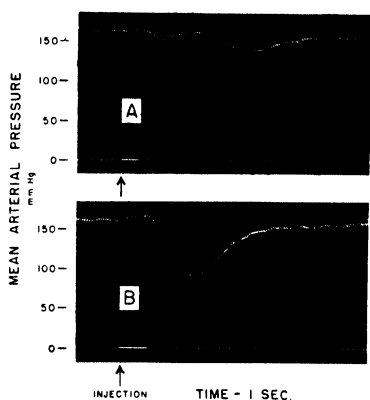


Fig. 1 (Dog 14, 11.4 kg.). MERCURY MANOMETER RECORDS showing effects of intravenous injection of mixture of 4 ml. of 20% NaCl with 16 ml. of 0.9% NaCl (curve A) and mixture of 4 ml. of 20% NaCl with 16 ml. of blood (curve B).

made into one of the external jugular veins at rapid rates (about 2.8 to 6.7 ml/second in experiments on dogs and cats; 1 to 3 ml/second in the experiments on rabbits). Volumes of fluid given on injection varied from 3 to 10 ml. for cats and rabbits, 15 to 30 ml. for dogs.

Frequently comparable volumes of either 0.9 per cent NaCl or blood mixed with 0.9 per cent NaCl were given as controls for temperature, volume and other side effects of the injection. The controls will not be discussed in detail, for they were either without effect on the mean arterial pressure, or they caused exceedingly brief and variable pressure alterations which were never greater than ± 15 mm. Hg.

RESULTS

Experiments on Dogs. Whereas the injection of saline-20 per cent NaCl mixtures produces a characteristic diphasic depressor response in all experiments, the administration of mixtures of blood with 20 per cent NaCl resulted in a rapid and frequently profound fall in blood pressure which had only a single phase. This is illustrated in figure 1, showing mercury manometer tracings from a typical experi-

ment. In this figure it may be seen that the fall in blood pressure after injection of a blood-20 per cent NaCl mixture occurred rapidly, having approximately the same latency as the primary depressor phase following injection of an equal volume of an equally concentrated solution of NaCl alone. The secondary phase of the depressor response caused by injection of hypertonic NaCl developed more slowly. In this experiment the fall in pressure after injection of a mixture of blood with hypertonic NaCl was greater than the maximum fall following administration of an equally concentrated saline-20 per cent NaCl mixture. These results are representative of the series of 9 experiments in which this comparison was made, although there was a single case in which the fall in pressure in the second phase of the response to hypertonic NaCl was greater than the decrease following injection of a corresponding blood-20 per cent NaCl mixture. Table 1 presents a summary of the findings obtained comparing the magnitude of the hypotensive responses to blood-20 per cent NaCl mixtures with the effects of hypertonic solutions alone.

TABLE 1. MAXIMAL DECREASE IN MEAN ARTERIAL PRESSURE FOLLOWING RAPID INTRAVENOUS INJECTION OF BLOOD-20% NaCl MIXTURES AND EQUALLY CONCENTRATED SOLUTIONS OF NaCl ALONE

SPECIES	NO. OF EXPER.	AVERAGE MAXIMAL FALL IN MEAN ARTERIAL PRESSURE					
		Blood-20% NaCl Mixtures		0.9% NaCl-20% NaCl Mixtures			
				Primary ¹ Phase		Secondary ¹ Phase	
				mm. Hg		mm. Hg	
		av	σ	av	σ	av	σ
Dogs.....	9	57	10	15	6	35	20
Rabbits.....	4	41	(7)	18	(8)		
Cats.....	6	71	17	70	15		

¹ Characteristic primary and secondary phases of hypotension following injection of hypertonic NaCl occurred only in dogs, not in rabbits or cats.

When blood-20 per cent NaCl mixtures were allowed to stand up to 20 minutes after preparation, and were subsequently injected intravenously, the resultant monophasic fall in blood pressure was somewhat less in magnitude than the hypotension caused by such mixtures administered within one or 2 minutes of preparation. This difference, observed in 3 experiments, was not great, however, and may not have been significant in view of the small number of observations.

Hamilton manometer records showed that the injection of mixtures of blood and 20 per cent NaCl was followed by a brief period in which the pulse pressure was irregularly reduced. Later the pulse pressure increased above the control level. In the absence of bradycardia, this indicated that vasodilation had occurred. As noted previously (4), a similar sequence of events is seen after administration of solutions of NaCl alone. The measured rise in pulse pressure occurred, however, at an earlier time when blood had been mixed with the hypertonic solutions prior to injection (table 1). Likewise, plethysmographic records showed an increase in hind foot volume following injections of blood-20 per cent NaCl mixtures. As shown in table 2, this rise in foot volume began within 18 to 20 seconds after injection, and thus preceded

the increase which resulted from administration of hypertonic NaCl alone. These data are consistent with the results of the mean arterial pressure measurements indicating that an interaction between blood and hypertonic NaCl occurs which causes a modification in the effects of the hypertonic agent.

Changes in heart rate following the injections were not especially striking, nor was it possible to distinguish with certainty the effects upon the heart rate of blood-20 per cent NaCl mixtures as compared with saline-20 per cent NaCl mixtures. In some experiments the heart rate did not vary by more than ± 15 per cent of the control value. In other cases, a transient irregularity and bradycardia were seen initially

TABLE 2. TIMING OF ALTERATIONS IN MEAN ARTERIAL PRESSURE, PULSE PRESSURE AND HIND FOOT VOLUME IN DOGS FOLLOWING INJECTIONS OF HYPERTONIC FLUIDS

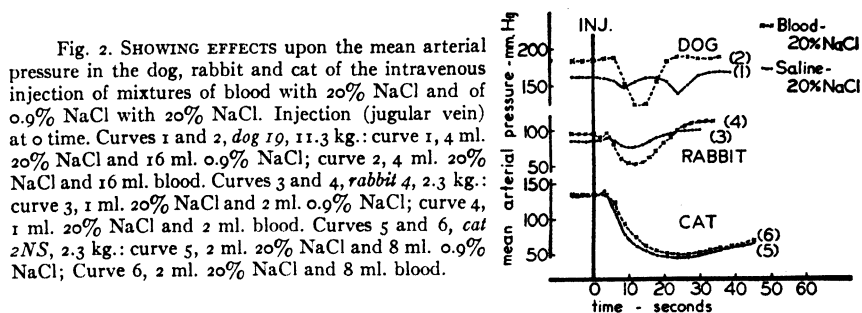
EXPER. NO.	LATENCY IN SECONDS OF FALL IN MEAN ARTERIAL PRESSURE			TIME IN SECONDS TO MINIMUM OF BLOOD PRESSURE CURVE		
	0.9% NaCl-20% NaCl Mixtures		Blood-20% NaCl Mixtures	0.9% NaCl-20% NaCl Mixtures		Blood-20% NaCl Mixtures
	Primary Phase	Secondary Phase		Primary Phase	Secondary Phase	
14	4.8	19.0	5.1	9.2	27.2	13.8
15	5.3	22.2	6.2	12.2	29.0	10.4
16	5.3	16.1	6.2	9.9	25.2	13.8
17	4.6	23.2	5.2	13.1	28.4	14.2
19	4.9	17.6	5.4	10.4	23.4	13.9
20	7.4	23.4	7.4	14.2	30.4	17.4
28	5.9	13.4	5.9	9.3	21.9	11.7
30	4.7	16.2	5.8	13.2	26.7	14.2
31	5.7	20.0	4.9	11.1	30.6	14.8
32	2.5	17.0	3.8	8.5	36.0	16.4
	LATENCY IN SECONDS OF INCREASE IN ARTERIAL PULSE PRESSURE		LATENCY IN SECONDS OF INCREASE IN HIND FOOT VOLUME			
15	31	25				
16	24	19.5				
31	18	16				
28				24		18
30				32		20

and in several experiments were followed by a moderate to marked tachycardia. The increased heart rate was approximately correlated, in time, with the arterial hypotension.

Experiments on Rabbits. In 4 experiments on anesthetized rabbits, the intravenous injection of saline-20 per cent NaCl mixtures caused a fall in blood pressure after a 10- to 26-second latent period. The hypotension was not characteristically diphasic, and frequently was of slight degree and brief duration. Injection of blood-20 per cent NaCl mixtures was followed by a decrease in mean arterial pressure of markedly greater intensity and usually of longer duration than the fall resulting from administration of NaCl alone. The latency of the hypotensive response following ad-

ministration of blood-20 per cent NaCl mixtures ranged from 13 to 18 seconds. Results of a typical experiment are illustrated in figure 2, and table 1 summarizes the findings on maximal changes in mean arterial pressure in the rabbit. It may be concluded from these findings that in the rabbit, as in the dog, interaction between hypertonic NaCl and blood plays a part in the development of hypotension following administration of blood-20 per cent NaCl mixtures. Alterations in heart rate were slight or negligible. Measurable slowing of the heart occurred in some experiments after injection of both blood-20 per cent NaCl mixtures and hypertonic NaCl alone. This transient bradycardia was occasionally succeeded by a brief tachycardia. The effects upon the heart rate of the two kinds of injections, in contrast to the effects upon mean arterial pressure, could not be definitely differentiated.

Experiments on Cats. Six experiments performed on cats, anesthetized with sodium pentobarbital, gave results which differed markedly from the findings in dogs and rabbits. It was noted that in the cat the intravenous injection of both saline-20 per cent NaCl and blood-20 per cent NaCl mixtures was followed, after a latent



period of 2.5 to 4.5 seconds, by a profound fall in blood pressure and bradycardia. The pattern of this response for the two injection fluids did not differ consistently either with respect to intensity or timing (fig. 2, table 1). This was found to be the case when mixtures of blood and 20 per cent NaCl were prepared and allowed to stand for 28 to 72 minutes before intravenous injection, as well as when they were injected within one to 5 minutes after preparation. Following vagotomy, the striking bradycardia was absent, and usually the hypotension resulting from both the blood-20 per cent NaCl and saline-20 per cent NaCl mixtures was reduced. These results gave no evidence for an interaction between blood and hypertonic NaCl in the development of the hypotensive response to injections of concentrated solutions.

DISCUSSION

The results described above show that, in the dog, anesthetized with sodium pentobarbital, the admixture of blood with hypertonic NaCl causes a marked qualitative, as well as quantitative, modification in the fall in mean arterial pressure following injection of the hypertonic agent. The qualitative alteration may be described as a shift in the timing of the depressor response so that the characteristic

primary and secondary depressor phases caused by pure hypertonic solutions are replaced by a single fall in pressure when blood-20 per cent NaCl mixtures are injected. Quantitatively, the effect of admixture of blood with 20 per cent NaCl is to increase the intensity of the hypotensive response. These highly constant results suggest that some interaction takes place between blood and hypertonic solutions. It seems probable, moreover, that if such an interaction takes place *in vitro*, it also occurs *in vivo*, and the overall effects of hypertonic solutions may be modified by this phenomenon. No clues are furnished by the data as to the nature of this postulated interaction, however, and further tests have been undertaken to investigate this problem. The results of these experiments will be presented in a separate communication.

The hypothesis that the hypotension is reflex in origin, as suggested by Gennari and Levi (1), has been demonstrated in a variety of ways to be untenable (2, 4, 5). Muirhead and his co-workers (2) suggested that hypertonic agents, after intravenous injection, may reach the myocardium first by direct diffusion from the cardiac chambers, and subsequently through the coronary circulation. This might account for the two phases of the hypotension. These workers cited as evidence for their hypothesis the fact that the fall in arterial pressure is monophasic rather than diphasic in character when the injection is made into the root of the aorta rather than on the venous side of the system, or when small injections are made directly into the coronary arteries. In view of our findings that admixture of blood with the hypertonic solution plays a critical role in determining the character of the depressor response, it seems possible that intra-aortic injections may allow for more rapid mixing of the hypertonic agent with blood than when the fluids are introduced on the venous side. Because of the sensitivity of myocardial activity to variations in the character and volume of its blood supply, it is difficult to compare directly results obtained from injections into the coronary arteries with those made at other vascular sites.

The circulatory effects of hypertonic solutions appear at the present time to be far more complicated than was at first believed. Although the nature of the interaction between concentrated NaCl solutions and the blood of dogs has not been defined, it seems quite clear that a similar interaction occurs in the case of rabbits following administration of strongly hypertonic solutions. On the other hand, no conclusive evidence for such an effect has been obtained in the case of the cat anesthetized with sodium pentobarbital. This species difference in response between dogs and rabbits, on the one hand, and cats on the other, is as yet unexplained but may, perhaps, prove useful in elucidating the more intimate mechanism of the cardiovascular effects of hypertonic solutions.

SUMMARY

The brief but profound hypotension which follows intravenous injection of hypertonic solutions in anesthetized dogs and rabbits may be markedly altered, with respect to both its severity and timing, by admixture of the hypertonic agent with homologous blood previous to injection. The findings suggest that an interaction between hypertonic agents and the blood itself may play an important role in the development of the hypotensive response. It has been found that the Nembutalized

cat differs from the dog and rabbit in that the response to intravenous injections of mixtures of blood with hypertonic solutions is not significantly different from the effects of hypertonic solutions of NaCl alone.

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MEASUREMENT OF EXTRACELLULAR FLUID BY MEANS OF A CONSTANT INFUSION TECHNIQUE WITHOUT COLLECTION OF URINE^{1,2}

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ANY substance occupying a fixed fraction of body water will approach a uniform or equilibrium distribution in that compartment under conditions where its rate of infusion and its clearance or specific rate of removal remain constant for an adequate period of time. After uniform distribution is attained, if the infusion is stopped and the total amount of substance present in the body is recovered quantitatively in the urine, the volume of distribution, V_s , can be calculated as the quantity recovered, R , minus the quantity contained in the renal dead space, D , divided by the equilibrium concentration, P_s :

$$V_s = \frac{R - D}{P_s} \quad \text{or} \quad V_s = \frac{R}{P_s} \quad \text{when } D \text{ is negligible.}$$

This is the basic principle in the use of inulin in measuring the volume of extracellular fluid in dog and man (1-3). It is the purpose of this paper to describe the extension of this principle to the determination of the volume of distribution of substances that cannot be quantitatively recovered in the urine.

The relationship between the volume of distribution of a substance, V_s , the renal clearance and the decrement in plasma concentration with time as utilized by Newman, Bordley and Winternitz (4) was modified to take into account any extrarenal disposal as follows:

$$(1) \quad S = V_s \cdot P,$$

where S is the amount of substance present in the body at any time t after equilibration between plasma and extravascular fluid, and P is the concentration of the substance throughout its volume of distribution.

The change in S with time will be equal to the rate of renal excretion UV and the rate of extrarenal disposal, E , as follows:

$$(2) \quad \frac{dS}{dt} = - \left(\frac{UV}{P} + \frac{E}{P} \right) P.$$

But

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$$(3) \quad \frac{UV}{P} + \frac{E}{P} = T_c.$$

where T_c is the total plasma clearance of the substance.

From equations 1-3, so long as T_c and V_s remain constant, it follows that:

$$(4) \quad \frac{dP}{P} = \frac{-T_c}{V_s} dt.$$

Integration between t and t' , where the corresponding plasma concentrations are P and P' , respectively, yields:

$$(5) \quad \ln P - \ln P' = \frac{T_c}{V_s} (t' - t)$$

$$(6) \quad V_s = \frac{T_c(t' - t)}{\ln P - \ln P'}.$$

This derivation assumes that during the period of removal of the substance, it remains homogeneously dispersed throughout its volume of distribution. Wherever the falling curve of plasma concentration is linear when plotted semi-logarithmically against time, the error involved in this assumption is not significant.

Thus, under conditions of constant total clearance, the volume of distribution of a rapidly diffusible substance is equal to the total clearance divided by the decrement with time (slope) of the logarithm of the plasma concentration.

METHODS

Human volunteers and trained unanesthetized female dogs were used. The human subjects were 6 normal men varying in age from 18 to 65 years. The dogs were adult mongrels ranging in size from 15 to 25 kg. Experiments were carried out in the 14- to 20-hour post-absorptive state with the animals lightly restrained on an animal board. The human subjects were allowed a light breakfast consisting of a glass of milk and buttered toast but were kept recumbent in bed. Water was given *ad libitum* and approximately 20 cc. of water per kilogram of body weight were given slowly before the infusion was started.

The volume of distribution of mannitol and in some cases, thiosulfate, p-aminohippurate and T_{125I} was determined in the following manner: A specimen of plasma was obtained for the blank determination; then, after a priming dose of the test substance, a sustaining infusion was given with a calibrated constant infusion pump. Following a suitable interval for equilibration, several blood samples were drawn from the femoral artery at 15- to 20-minute intervals. The infusion was stopped and several more samples of blood were taken at intervals for one to 2 hours. At equilibrium the amount of substance infused per unit time (the pump rate of flow times the concentration in the infusate), designated as I , must equal the amount of substance eliminated per unit time by all routes (renal UV plus extrarenal E).

The total plasma clearance of the substance T_c is calculated by dividing the rate of infusion I by the equilibrium plasma concentration P and thus includes and corrects for loss by metabolism. The decrement with time of the logarithm of plasma concentration is calculated from the post-infusion plasma concentrations. This procedure is illustrated schematically in figure 1.

Mannitol was determined by the method of Corcoran and Page (5), p-aminohippurate by the method of Bratton and Marshall (6) as modified by Smith, Finkelstein, Aliminosa, Crawford and Graber (7), thiosulfate by the method of Newman, Gilman and Philips (8) and T_{125I} was determined in the Beckman spectrophotometer, model DU, using a light path of 10 mm. at a wave length of 625 mμ.

RESULTS

Twelve technically satisfactory experiments were made in 6 dogs and 6 studies were made in 6 normal men. In all these experiments the logarithm of the plasma concentration of mannitol, thiosulfate, p-aminohippurate and T_{1824} bore a linear relationship to time after the infusion was stopped. The mannitol, thiosulfate and p-aminohippurate spaces as determined by the above method in the dog and man are shown in tables 1 and 2, respectively. The mannitol space averaged 19.2 per cent of body weight in the dog with a range from 16.3 to 21.4 per cent. In man the mannitol space averaged 17.3 per cent of body weight with a range from 15.4 to 19.9 per cent. To emphasize the independence of plasma clearance and plasma concentration,

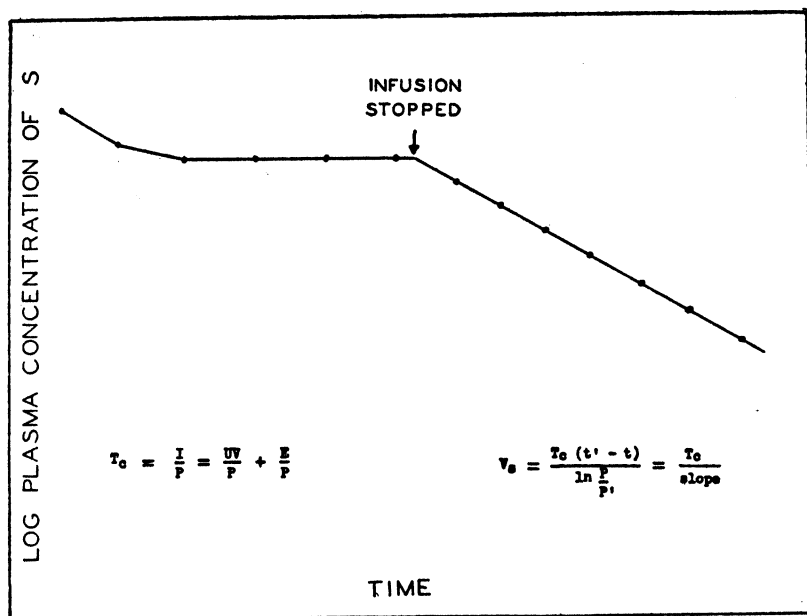


Fig. 1. IDEAL TIME-CONCENTRATION CURVE for calculating volume of distribution of a substance from the total clearance and the rate of disappearance of the substance from plasma.

an experiment was carried out in a dog in which the plasma mannitol concentration was held temporarily constant by an infusion, then allowed to fall for an hour, and held temporarily constant at a lower concentration by a second infusion. Determinations of the total mannitol clearance at plasma concentrations of 128 mg. per cent and 63 mg. per cent were 54.8 cc/minute and 55.6 cc/minute, respectively.

The average of 4 measurements of the thiosulfate space in the dog was 17.0 per cent of body weight; and 2 measurements of the thiosulfate space in man were 15.7 and 19.5 per cent of body weight. Two measurements of the p-aminohippurate space in each of 2 dogs and in each of 2 men were 27.5 and 26.6 per cent of body weight and 28.5 and 26.2 per cent of body weight, respectively.

The T_{1824} space was measured by the above method and by the single injection technique in a human subject and in one dog. In the human subject the clearance of T_{1824} was 3.84 cc/minute and the T_{1824} space by the infusion method was 5.94 per cent of body weight as compared to 5.0 per cent by the single injection method. In the dog the clearance of T_{1824} was 0.94 cc/minute and the T_{1824} space by the infusion method was 7.9 per cent of body weight as compared with 7.4 per cent by the single injection method.

DISCUSSION

The infusion-slope method here described was developed in order to measure the extracellular water volume with solutes which may be removed from the body otherwise than in the urine even when the actual route of removal is unknown.

TABLE 1. TOTAL PLASMA CLEARANCE AND VOLUMES OF DISTRIBUTION OF MANNITOL, THIOSULFATE AND P-AMINOHIPPURATE IN DOGS

EXPER. NO.	DOG	WT.	EQUILIBRIUM CONCENTRATION IN PLASMA			TOTAL PLASMA CLEARANCE			VOLUME OF DISTRIBUTION		
			Mannitol	Thio-sulfate	PAH	Mannitol	Thio-sulfate	PAH	Mannitol	Thio-sulfate	PAH
		kg.	mg. %	mg. %	mg. %	cc/min.	cc/min.	cc/min.	l.	l.	l.
1	I	16.3	125			52.3			3.23		
2	I	15.4	115.4			61.4			2.10		
3	2	23.6	128			54.8			4.78		
4	2	23.1	131			59.5			4.75		
5	2	21.6	125.8	50.6		47.1	56		4.63	4.02	
6	3	16.0	61	52.3		52.8	65.5		2.66	2.36	
7	3	16.3			2.38			191			4.46
8	4	24.0	92	32.7		80.6	111		4.16	4.32	
9	4	22.5			1.56			350			6.00
10	5	15.5	79.8			45.4			3.00		
11	6	22.8	191	75.4		55	57.8		4.21	3.73	

This method enjoys the advantage of freedom from error due to the renal dead space and to changing blank excretion in the urine.

The values obtained for the volume of distribution of mannitol and thiosulfate indicate that these solutes are confined to the extracellular space, in accordance with the conclusions of others (4, 8-11). The larger volume of distribution of p-aminohippurate demonstrates that this solute enters tissue cells, which circumstance excludes the use of this substance as an indicator of the extracellular fluid volume.

Although inulin probably does not enter the intracellular compartment, it cannot be employed as an indicator of the extracellular space by the infusion-slope method. Because inulin is a slowly diffusing substance (12), the falling curve of plasma inulin concentration plotted semi-logarithmically against time is never linear, even after equilibrium has been established between plasma water and interstitial fluid (3).

The use of this method makes possible the measurement of the T_{1824} space by

means other than the direct determination of the dye volume dilution following a single injected dose. Application of this method to the measurement of the clearance and volume of distribution of T_{1824} is rendered feasible by the fact that T_{1824} disappears from plasma at a rate proportional to concentration (13, 14).

The increment in osmotic pressure of the extracellular fluid due to the presence of mannitol, thiosulfate or p-aminohippurate is negligible in the amounts employed in these experiments.

When the clearance of a substance changes, as indicated by lack of a constant plasma concentration during infusion and/or by failure to obtain a linear decrement with time of the logarithm of plasma concentration after the infusion is stopped, the conditions required for measurement by this method are not fulfilled. Experiments performed under circumstances of changing clearance are not valid, and no calculation of volume of distribution is justified in such cases.

TABLE 2. TOTAL PLASMA CLEARANCE AND VOLUMES OF DISTRIBUTION OF MANNITOL, THIOSULFATE AND P-AMINOHIPPURATE IN MAN

EX- PER.	SUBJECT	WT.	EQUILIBRIUM CONCENTRA- TION IN PLASMA			TOTAL PLASMA CLEARANCE			VOLUME OF DISTRIBUTION		
			Mannitol	SrO ₃	PAH	Mannitol	SrO ₃	PAH	Mannitol	SrO ₃	PAH
		kg.	mg.-%	mg.-%	mg.-%	cc/min.	cc/min.	cc/min.	l.	l.	l.
1	S. S.	68.0	90		2.81	101.7		573	11.72		19.4
2	P. O'D.	65.5	111.7			101.8			10.2		
3	T. F.	60.0	137.4		2.29	123.8		622	11.2		15.7
4	R. B.	62.3	100.1	41.1		116	161.7		9.57	9.78	
5	T. H.	62.7	93.3	46.8		124	169.5		12.5	12.25	

SUMMARY

The volume of distribution of a substance may be determined regardless of the route of removal of the substance from the body, *a*) if it is freely and rapidly diffusible, and *b*) if it is removed from the body water compartment in which it is dissolved at a rate proportional to its concentration.

A constant infusion was used to determine the overall or total plasma clearance of mannitol, p-aminohippurate, thiosulfate and T_{1824} , following which the infusion was stopped, and the decrement with time of plasma concentration was observed. The volume of distribution of each of these substances was calculated as the total plasma clearance divided by the post-infusion decrement with time (slope) of the natural logarithm of plasma concentration, thus correcting for any metabolic or other extrarenal disposal.

The mannitol space averaged 19.2 per cent of body weight in the dog and 17.3 per cent of body weight in man. The thiosulfate space averaged 17.0 per cent of body weight in the dog and 2 measurements of the thiosulfate space in man were 15.7 and 19.5 per cent of body weight. The p-aminohippurate spaces were 27.5 and 26.6 per cent of body weight in 2 dogs and 28.5 and 26.2 per cent of body weight in 2 men. Measurements of the T_{1824} space in a human subject and in a dog were 5.94 and 7.9 per cent of body weight, respectively.

The author is indebted to Dr. David Schachter for suggesting the study of the clearance and volume of distribution of T_{1824} by this method and for the data obtained with T_{1824} in the dog.

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MOVEMENT OF INULIN BETWEEN PLASMA AND INTERSTITIAL FLUID^{1, 2}

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THE movement of inulin between the plasma and interstitial fluid was studied in the following manner: The inulin space was determined repeatedly in each subject using the constant infusion technique described elsewhere (1). On another occasion each subject received a single intravenous injection of 20 to 75 cc. of 10 per cent inulin from a calibrated syringe, following which serial samples of venous blood and urine were collected for 24 hours. Before the injection a control blood and timed urine sample were obtained for determination of the plasma blank concentration, B_0 , and the rate of excretion of inulinoid blank, U_0V , which quantities were subtracted respectively from plasma concentrations and inulin excretion in subsequent urine periods. The inulin concentration of all samples was determined by Harrison's modification (2) of the method of Alving, Rubin and Miller (3). No correction was made for plasma water content.⁴ Plasma volume was measured with T_{1824} from a single venous sample taken 10 minutes after injection of the dye in the human subjects (4) and by extrapolation to zero time of the arterial time-concentration curve in the dogs. T_{1824} concentrations were determined in the Beckman spectrophotometer, model DU, at a wave length of 625 $m\mu$ using cuvettes with a light path of 10 mm.

RESULTS

Results of these experiments were analyzed as illustrated schematically in figure 1. At any time after the injection, t , the amount of inulin present in the interstitial fluid, Z , will equal the total injection dose, D , less the quantity excreted in the urine, UV , and the quantity left in the blood, $P_{WIN} \cdot P_{WV}$ where P_{WIN} and P_{WV} represent the plasma water inulin concentration and plasma water volume, respectively. The volume of interstitial fluid, I_v , equals the volume of extracellular fluid, ECF , less the plasma water volume, P_{WV} . Therefore when Z is divided by I_v , the quotient represents the concentration of inulin in the interstitial fluid if the inulin were evenly dispersed throughout that compartment. Since uniform dispersion does not occur after a single injection, we refer to the quotient, Z/I_v , as the virtual concentration of inulin in interstitial fluid.

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⁴ For precise application of this method it is necessary to correct UV for the renal delay time and to determine the concentration of plasma protein in order to obtain the concentration of inulin in plasma water.

Data obtained from a typical experiment are illustrated in figure 2. The solid line shows the falling curve of plasma concentration and the dotted line shows the rising curve of cumulative excretion in the urine, UV . The virtual concentration of inulin in interstitial fluid, Z/I_v , is then calculated in the manner described and plotted,

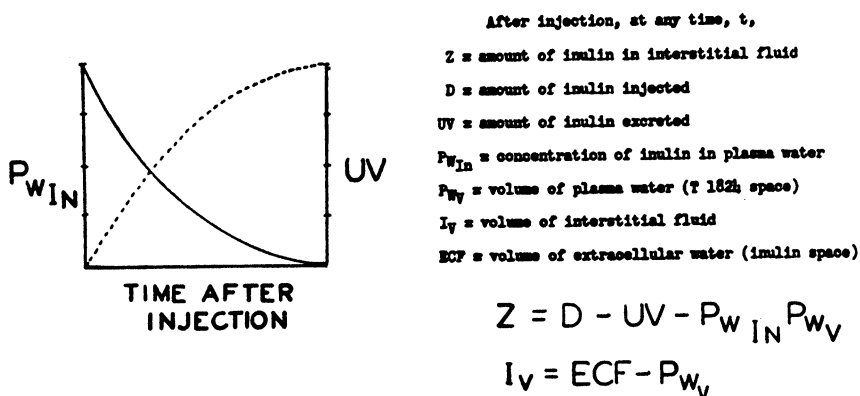


Fig. 1. METHOD for determining amount of inulin in the interstitial compartment after a single intravenous injection.

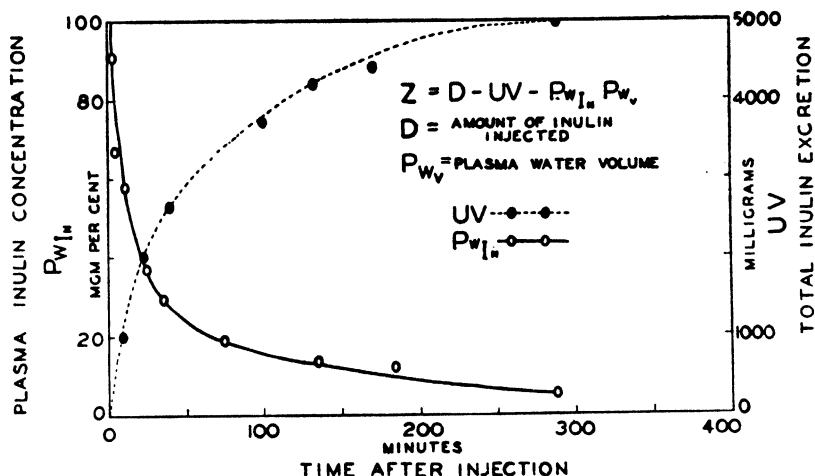


Fig. 2. CONCENTRATION IN PLASMA AND TOTAL CUMULATIVE EXCRETION after a single intravenous injection of inulin in a normal human subject.

simultaneously with the plasma inulin concentration, against time (fig. 3). Here the solid line again represents the falling curve of plasma concentration and the dotted line represents either the total amount of inulin in the interstitial fluid, Z (right) or the virtual concentration of inulin in the interstitial fluid, Z/I_v (left). It is seen that the plasma is quickly depleted of inulin by renal excretion and by rapid movement into the interstitial fluid. At time A the plasma-interstitial fluid gradient is reversed,

and inulin begins to move back into the blood stream, the higher plasma concentration at this point indicating that it has saturated only those parts of the interstitial

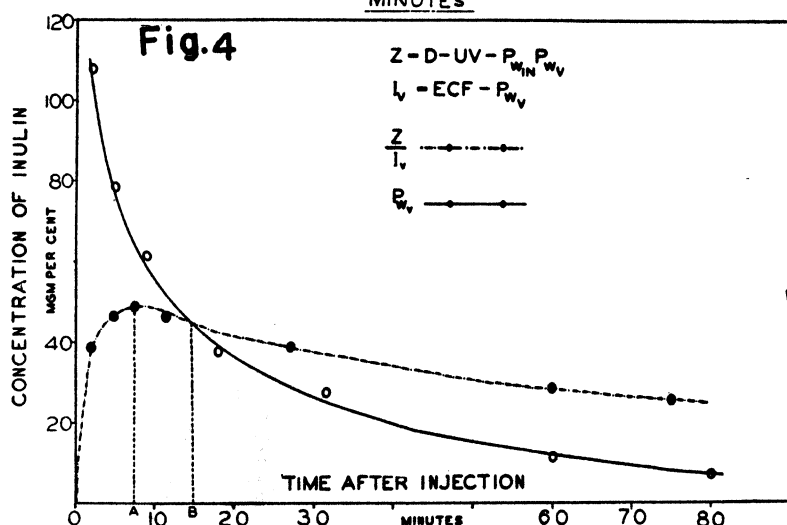
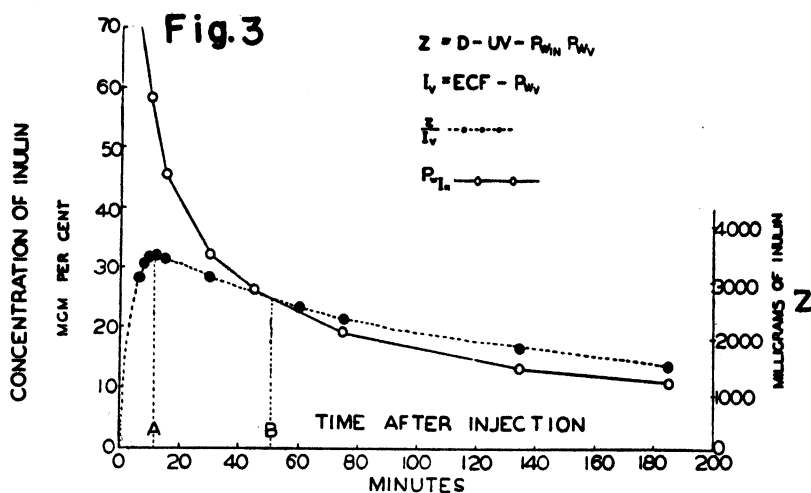


Fig. 3. CONCENTRATION IN PLASMA, virtual concentration in interstitial fluid (left) and total amount of inulin in interstitial fluid (right), following a single intravenous injection in a normal human subject. The values were calculated from data in figure 2.

Fig. 4. CONCENTRATION IN PLASMA and virtual concentration in interstitial fluid of inulin following a single intravenous injection in a dog.

fluid most available to interchange with the blood. It is only at point *B* that a plasma sample is representative of inulin concentration throughout its volume of distribution and may be used to calculate the inulin space:

$$\frac{\text{Dose—amount excreted to time } B}{\text{Plasma concentration at time } B}$$

Data from a similar experiment in a dog are shown in figure 4. The time required to reach point *A* was 12, 22.5 and 5 minutes in 3 human subjects and 7.5 and 10 minutes in 2 dogs. The time required to reach point *B* was 51, 33 and 9 minutes in the human subjects and 14.5 and 25 minutes in the dogs.

The data presented here illustrate the fact that the mixing of inulin throughout the extravascular compartments occurs relatively slowly as would be expected from the low diffusion coefficient (5, 6). It is evident therefore that the inulin space can be measured accurately only when a constant infusion has been maintained for a sufficiently long period to insure homogeneous dispersion of inulin throughout the volume of distribution. Recently the rate of equilibration of inulin between plasma water and extravascular fluid has been studied by Robson, Ferguson, Olbrich and Stewart and formulas have been adduced for correction of the measurement of the inulin clearance during a period of changing plasma concentration (7).

SUMMARY

The movement of inulin between plasma and interstitial fluid was studied by measuring the rate of disappearance of inulin from plasma, the rate of excretion of inulin in the urine, the plasma volume and the inulin space. After a single intravenous injection of inulin, true plasma-interstitial fluid equilibrium never occurs, and virtual equilibrium is momentary. The plasma-interstitial fluid inulin concentration gradient reversed itself in 5, 12 and 22.5 minutes in 3 human subjects, and in 7.5 and 10 minutes in 2 dogs. Identity of plasma inulin concentration and virtual interstitial fluid inulin concentration occurred in 9, 33 and 51 minutes in the human subjects and 14.5 and 25 minutes in the dogs. These times differ too greatly to permit their use in a single injection technique for determination of the inulin space.

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CHLORIDE EXCRETION DURING OSMOTIC DIURESIS IN THE DOG

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IN THE recently reported experiments on dehydration from this laboratory it was noted that in diuresis dehydration (occasioned by injection of 50% sucrose) the nature of the fluid changes depended to a large extent on the degree and character of the electrolyte loss during the diuresis (1). This led us to study the factors involved in the excretion primarily of extracellular electrolyte during osmotic diuresis. Since the total amount of sodium excreted was similar to the chloride value (table 1), attention was limited to the chloride ion. In order to broaden the scope of this study, other non-electrolyte osmotic diuretics besides sucrose were also used, namely 10 per cent urea, 50 per cent glucose and 50 per cent sorbitol.

Through measurement of the glomerular filtration rates it was possible to evaluate the amounts of chloride filtered, excreted and reabsorbed during the diuresis. The effect of changes in serum chloride concentration on the excretion of chloride ion during diuresis was evaluated in animals made salt-deficient by previous sucrose injections and low salt diet (2) or in which serum chloride levels were elevated by addition of sodium chloride to the perfusate. In order to see if chloride excretion during osmotic diuresis can be easily modified, two substances reported to alter renal excretion of chloride, namely pituitrin and desoxycorticosterone, were given in conjunction with the injection of 50 per cent sucrose. These several approaches form the basis of this study.

PROCEDURE

This study, made on 8 unanesthetized adult female dogs can be subdivided into two phases. The dogs were first subjected to tests carried out in the manner described in a previous paper (1), in order to establish the relation of chloride excretion to that of sodium and potassium, and to establish the relation of chloride excretion to speed of injection and varying amounts of hypertonic solution.

The second phase was a study of the creatinine clearance as a measure of glomerular filtration rate. Food was removed at 5:00 P.M. of the day preceding the experimental study but water was permitted *ad libitum*. At the start of the experiment the following day each dog was given 200 mg/kg. of creatinine, divided into a subcutaneous and intravenous dose. An indwelling catheter was inserted and 20 minutes later, after washing the bladder with distilled water, the control period was started. Immediately following the completion of the two control urine collection periods

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(20 to 30 minutes each) a continuous intravenous infusion of 50 per cent sucrose was started in a small vein of the hind leg. The infusion was given by a mechanical pump at a constant rate of 2 cc/minute, for a total dose of 12.5 cc/kg. of body weight. For the sake of uniformity, once the dosage of diuretic agent for a dog had been determined, the same amount was used in subsequent experiments on that dog regardless of minor fluctuations in weight. During the infusion urine collections were made as follows: the first sample, 15 minutes following the last control bladder washing, all others at 10-minute intervals, as long as infusion continued. At the conclusion of the injection 2 to 3 additional collections were made at 15- to 20-minute intervals. Venous blood samples for determination of plasma chloride and creatinine levels were taken at regular intervals from the jugular vein.

TABLE 1. URINE BLOOD CHANGES FOLLOWING INTRAVENOUS INJECTION OF 250 CC. 50 PER CENT SUCROSE SOLUTION (DOG, MIDNIGHT)¹

TIME	WEIGHT	PLASMA PROTEINS	BLOOD H ₂ O	HEMATOCRIT	SERUM Na	SERUM Cl	SERUM K
	Kg.	%	%	%	mEq.	mEq.	mEq.
Control 4 p.m.	14.35	6.3	80.2	40.8	144.5	105.5	3.76
<i>Diuresis</i>	After sucrose	976 cc.					
Urine Loss	Na	63.2 mEq.					
	Cl	63.0 mEq.					
	K	6.4 mEq.					
<i>Dehydration</i> 9 a.m.	13.12	8.45	75.9	48.9	158.0	109.0	4.26
<i>Drinking ad lib.</i>	Intake	920 cc.					
Urine Loss	Cl	0.2 mEq.					
<i>Hydration</i>	13.73	6.8	82.5	44.5	132.6	95.5	3.54

¹ Urine loss of electrolytes shows that amount of sodium excreted during the diuresis is equal to the chloride loss.

The infusion fluids used in these experiments included 50 per cent sucrose, 50 per cent sorbitol, 50 per cent glucose and 10 per cent urea and all were given in the same manner and in the same dosage, namely 12.5 cc/kg. In order to maintain adequate plasma creatinine levels the infusion fluids also contained one mg. of creatinine/cc.

In a second series of experiments chloride excretion was studied during sucrose diuresis at both increased and decreased chloride levels. Increased plasma chloride levels were obtained by adding enough salt to the infusion fluid to make it equivalent to 10 per cent sodium chloride. This raised plasma chlorides by 5 to 7 mEq/l. Low chloride levels were obtained by successive sucrose diuresis and maintaining the animals on a salt-poor diet (2). In this manner serum chlorides as low as 85 mEq/l. were obtained.

In a third series of experiments the animals were given either desoxycorticosterone² or pitressin³ in conjunction with 50 per cent sucrose infusion. The desoxycorticosterone was given in 2 equal doses, the first dose 18 to 24 hours and the second 3 to 4 hours before the first control period. Individual doses ranged from 5 to 20 mg. In one experiment 20 mg. were given 72 hours and 20 mg. at 24 hours prior to taking the first control urine sample. In the pitressin experiments, 10 pressor units were given subcutaneously at the time of the creatinine priming dose, and an additional 10 pressor units were given approximately 10 minutes after beginning the intravenous injection of 50 per cent sucrose.

Creatinine determinations on diluted urines and sodium tungstate plasma filtrates were made by treating with alkaline picrate solution according to the method of Folin and Wu (3) and reading in a Coleman spectrophotometer at 510 m μ . Urine

TABLE 2. CHARACTERISTIC URINARY FINDINGS DURING INTRAVENOUS INJECTION OF 50 PER CENT SUCROSE IN THE DOG^{1, 2}

URINE SAMPLE	A TOTAL TIME	B FILTRATION RATE	C CREATININE U/P RATIO	D URINE FLOW	E PLASMA Cl	F URINE Cl	G Cl EXCRETED	H SPECIFIC GRAVITY
		cc/min.		cc/min.	mEq/l.	mEq/l.	mEq/min.	
1	0-28	39.7	101.0	.39	110.0	11.68	.005	
2	28-53	41.2	171.4	.24	110.0	14.25	.003	
3	53-67	44.3	38.5	1.1	109.5	13.22	.015	
4	67-76	40.0	10.5	3.8	109.0	40.21	.152	1.032
5	76-85	45.0	7.6	5.9	109.0	50.45	.297	1.024
6	85-94	41.7	6.4	6.5	109.0	55.58	.358	1.026
7	94-104	40.0	5.8	6.8	109.4	56.43	.384	1.028
8	104-114	35.7	7.0	5.1	110.2	51.30	.262	1.032
9	114-125	41.7	10.2	4.1	111.0	39.33	.161	1.036
10	125-139	40.0	12.3	3.2	111.5	41.04	.132	1.048

¹ Dog 30, February 17, 1947, continuous infusion of 110 cc. of 50% sucrose at the rate of 2 cc/minute.

² Samples 1 and 2 represent control periods. Samples 3-8 represent period of injection of the 50% sucrose.

chlorides were determined by a modified Volhard-Harvey method (4) and plasma chlorides by the dye adsorption method of Saifer and Kornblum (5). All analyses were carried out in duplicate.

RESULTS

Effect of Injection of Osmotic Agents on Chloride Excretion. The effects of osmotic diuresis on chloride excretion were studied in 8 experiments with 50 per cent sucrose, 4 experiments with 50 per cent glucose, 4 experiments with 50 per cent sorbitol and 4 experiments with 10 per cent urea. The results of a typical experiment with 50 per cent sucrose are shown in table 2. During the diuresis there was an in-

² The desoxycorticosterone used in these experiments was furnished through the courtesy of Hoffmann-La Roche, Inc.

³ The pitressin used in these experiments was furnished through the courtesy of Parke, Davis and Company.

crease in the absolute amount of chloride excreted per unit time (Col. G). This held true for all experiments and all types of osmotic agents used. When we examine the urinary output (Col. D) we find the increased chloride excretion appears to vary as

TABLE 3. TYPICAL CALCULATIONS ON CHLORIDE REABSORPTION DURING SUCROSE DIURESIS^{1, 2}

SAMPLE	PERIOD	TOTAL TIME	FILTRATE RATE	URINE FLOW		PLASMA Cl CONC.	Cl EXCRETED	Cl EXCRETED	Cl FILTERED	Cl REABSORBED	FILTERED Cl REABSORBED
				Vol.	cc/min.						
	min.	min.	cc.	cc.			mEq.	mEq/min.	mEq/min.	mEq/min.	%
1	28	0-28	40.8	29	1.0	97.5	.086	.003	3.99	3.99	99.9
2	31	28-59	39.6	15	0.5	97.5	.070	.002	3.86	3.86	99.9
3	16	59-75	41.9	17	1.1	96.8	.096	.006	4.05	4.04	99.8
4	10	75-85	35.7	22	2.2	96.3	.122	.012	3.44	3.43	99.7
5	10	85-95	39.1	31	3.1	95.8	.308	.031	3.74	3.71	99.2
6	10	95-105	41.8	41	4.1	95.5	.795	.080	4.00	3.92	98.0
7	10	105-115	42.7	57	5.7	95.5	1.462	.146	4.08	3.93	96.2
8	10	115-125	42.5	65	6.5	95.5	1.866	.187	4.06	3.87	95.4
9	15	125-140	40.5	76	5.1	95.5	1.853	.124	3.87	3.75	96.9
10	15	140-155	37.3	55	3.7	95.5	1.044	.069	3.57	3.50	98.1

¹ Dog 964, 130 cc. 50% sucrose, June 11, 1947. Dog partly demineralized on salt-free diet.

² Samples 1 and 2 represent control periods. Samples 3 through 9 represent periods during which 50% sucrose was infused intravenously. Note that the percentage of filtered chloride reabsorbed decreases as the urine flow increases, lowest reabsorption obtaining at highest urine flow.

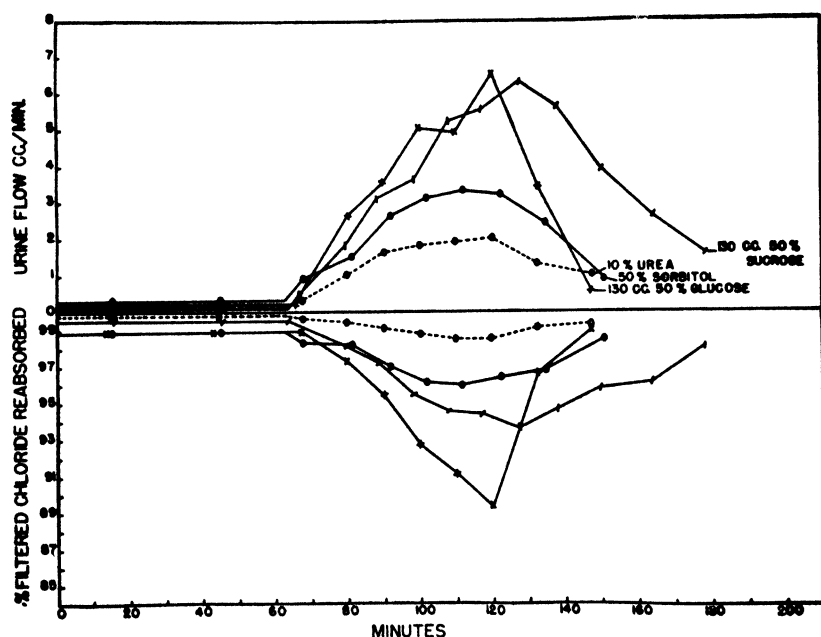


Fig. 1. COMPARISON of urine flow and simultaneous chloride reabsorption during diuresis occasioned by various osmotic agents in the same dog. Reabsorption decreases as urine flow increases, lowest reabsorption occurring at highest urine flows.

the urine flow and that in all experiments maximal chloride excretion occurred at the height of diuresis. Urinary chloride concentrations rapidly changed from the values found during control periods to values within a rather limited range despite large changes in urine flow (Col. *F*). The serum chloride did not vary in any instance more than 3 to 4 mEq. showing a tendency to drop during the period of injection and to rise slightly thereafter (Col. *E*). The specific gravity of the urine fell during the injection and began to rise after its completion (Col. *H*).

Early in the experiments it became evident that changes in filtration rate were not per se responsible for the increased chloride excretion since this occurred in

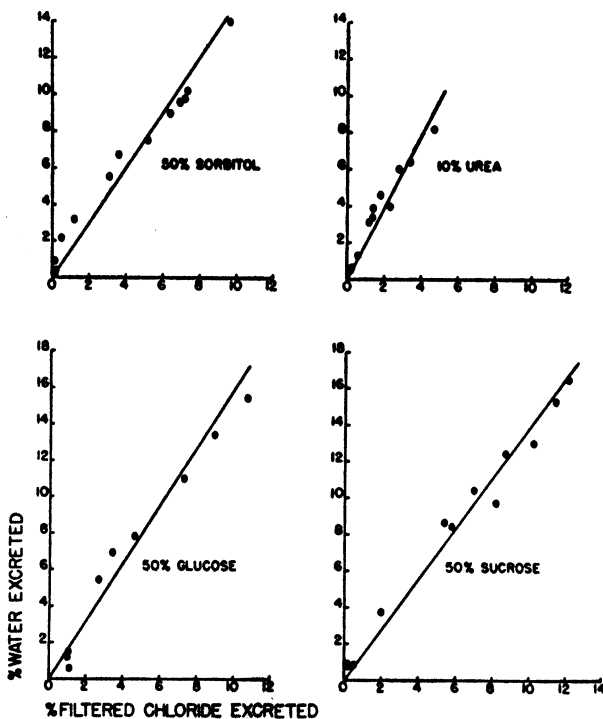


Fig. 2. STRAIGHT-LINE RELATIONSHIP between percentage of filtered water excreted and percentage of filtered chloride excreted in the same dog for all diuretic agents used.

absence of any change. For example in table 2 at the point of maximum chloride excretions period 6 and 7, the filtration rate was 41.7 and 40.0 cc/minute which was similar to the control periods where the rate was 39.7 and 41.2. In those experiments where the filtration did change there was no apparent alteration in the pattern of chloride excretion. Therefore, attention was directed towards the factor of reabsorption (table 3). Here it was found that as urine flow increased the per cent of filtered chloride being reabsorbed decreased, the lowest point in reabsorption occurring at the time of maximum urine flow. Analysis of the data showed that this phenomenon held true for all 4 types of osmotic diuretics used (fig. 1). Further study of this relationship between decreasing reabsorption of chloride and increasing urine flow

showed that when the per cent filtered chloride excreted was plotted against the per cent filtered water excreted, and an essentially straight-line relationship obtained. This was the case with all 4 diuretic agents employed (fig. 2). It was evident that in this type of diuresis the extent of the chloride loss was dependent on the extent of the diuresis.

Effect of Injection of Osmotic Agents on Chloride Excretion in Animals with Elevated or Decreased Plasma Chloride Levels. Since increased chloride excretion during osmotic diuresis was found to be independent of the type of osmotic agent used and since the phenomenon was shown to be directly related to urine flow,

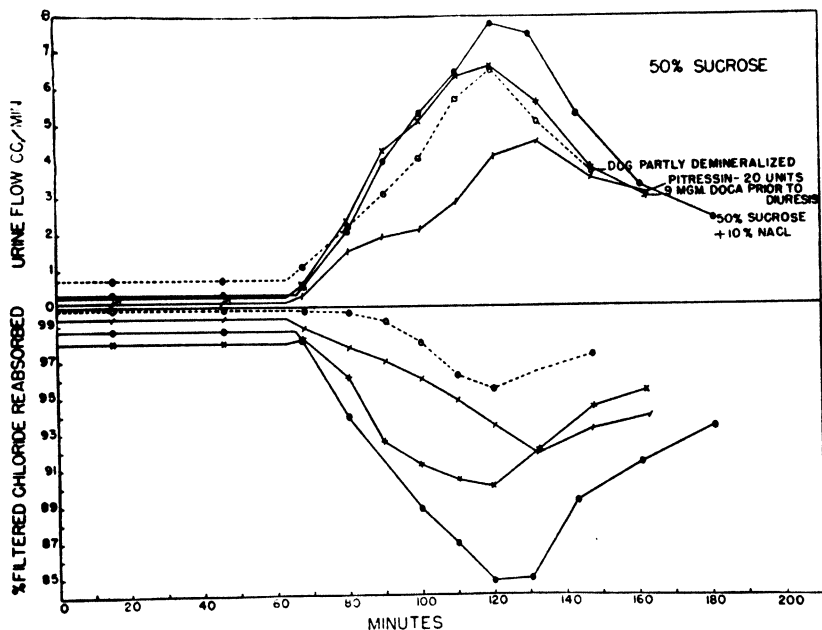


Fig. 3. COMPARISON of urine flow and simultaneous chloride reabsorption during osmotic diuresis in the same dog, plasma chloride levels of which were changed, and when prepared with desoxycorticosterone or with pitressin. In all situations reabsorption decreased as urine flow increased, lowest reabsorption occurring at highest urine flow.

attention was now directed to see whether or not this relationship would be altered by changes in the plasma chloride level. Accordingly, 7 experiments were performed at low plasma chloride levels (103–85 mEq/l.), and 3 experiments at elevated plasma chloride levels (116 and 117 mEq/l.). An increased chloride excretion obtained in all experiments, the maximal excretion coinciding with the height of diuresis (fig. 3). When the data was plotted as percentage of filtered chloride against percentage of filtered water excreted the same straight-line relationship was obtained as in previous experiments, showing that changes in plasma chloride levels had not affected this relationship (fig. 4). However, though the straight-line relationship between percentage of filtered chloride excreted and percentage of filtered water excreted was unaltered by change in plasma chloride concentration, the latter did alter the ratio between the water and chloride that was excreted (fig. 5). As plasma chloride level

is decreased, the ratio is increased, resulting in low urine chloride concentrations due to a relatively greater reabsorption of chloride than of water. The reverse effect obtains for an increase in plasma chloride level, i.e. a decreased ratio resulting in increased urinary chloride concentrations. In other words, the data of these experiments would indicate that during osmotic diuresis, there exists at a given plasma chloride level, a fixed water: chloride excretion ratio.

Effect of Injection of Osmotic Agents on Chloride Excretion in Animals Treated with Desoxycorticosterone Acetate or Pitressin. The straight-line relationship between

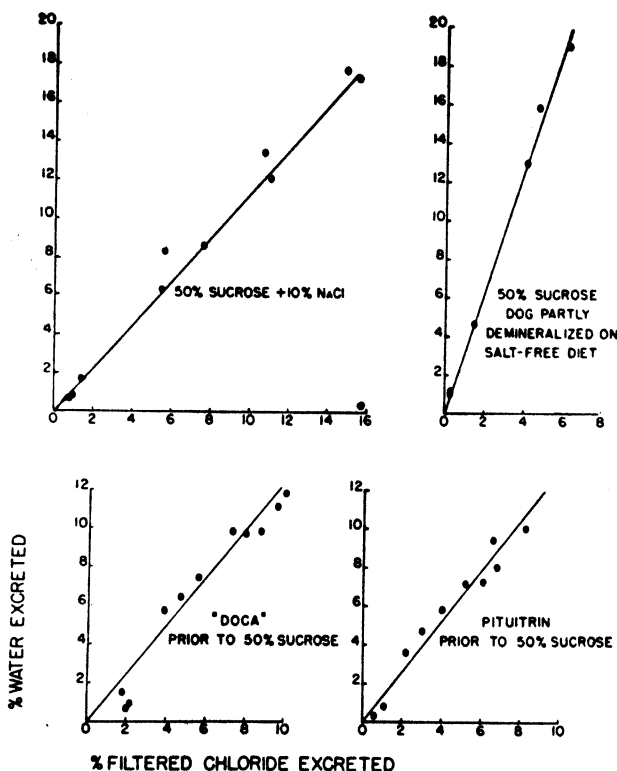


Fig. 4. STRAIGHT-LINE RELATIONSHIP between percentage of filtered water excreted and percentage of filtered chloride excreted in the same dog at high and low plasma chloride levels and when prepared with desoxycorticosterone acetate or pitressin.

the percentage of filtered water excreted and percentage of filtered chloride excreted was unaffected by desoxycorticosterone or pitressin. Apparently these agents are not capable of altering the chloride reabsorption during osmotic diuresis. In fact in the experiments illustrated in figure 5 there is no indication that they affected in any way the reabsorption of chloride under these conditions. However, there was the suggestion that desoxycorticosterone did in some instances change the ratio between percentage of chloride excreted and the percentage of water excreted, thus shifting

the curve to the left as was observed in the low salt dogs. It is curious that in the experiments recorded in figure 3 the desoxycorticosterone during the control periods did not display the usual chloride-retaining effect. Similarly the animal did not respond to pitressin during controls by showing an increased chloride output. This reversal of effects was not observed in other animals studied with these agents.

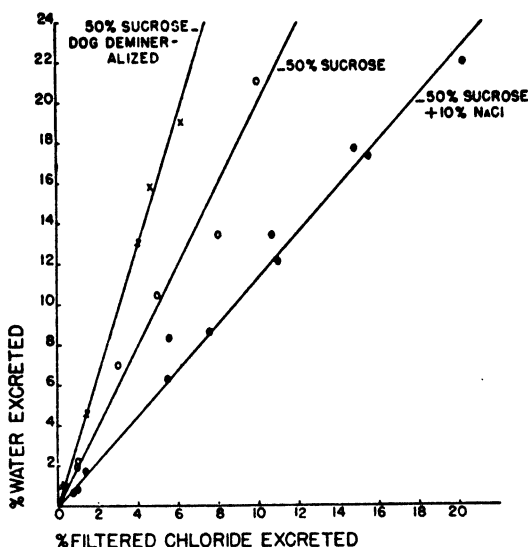


Fig. 5. RELATIONSHIP between percentage of filtered water excreted and percentage of filtered chloride excreted at different plasma chloride levels in the same dog (high plasma Cl, 115 mEq/l., normal 110 mEq/l., dimineralized 87 mEq/l.). Although a straight-line relationship obtained with either high, normal or low plasma chloride levels, the ratios varied so that low urinary chloride concentrations occurred at low plasma levels and the reverse at high plasma chloride levels.

DISCUSSION

It has been shown by several investigators that the diuresis brought about by glucose (6, 7), sucrose (2) and urea (8) is attended by increased urinary excretion of chloride ion. The present experiments confirm this finding and extend it to include sorbitol. It would appear then that this is a phenomenon occasioned by several non-electrolytic diuretics and their urinary excretion pattern might well be ascribed to their osmotic effect in the tubules. Rapoport, West and Brodsky (9), using a number of osmotic diuretics including those used in this study, found a similar straight-line relationship between urine flow and chloride loss in hydropenic man. The question arises as to whether this phenomenon of increased chloride excretion might also be extended to electrolytic osmotic agents. Lotspeich (10) attempts to explain the depression of sulphate reabsorption by sodium chloride as due to osmotic factors. Goudsmit (11) and associates studied in dogs the diuresis induced by infusion of 10 per cent sodium sulfate. Recalculation of their data shows that an essentially straight-line relationship exists between the percentage of filtered water excreted and the percentage of filtered chloride excreted during the diuresis. However Rapoport and associates (9) found in man that sodium sulfate diuresis did not produce the same straight-line relationship between chloride loss and urine flow that occurred after such substances as sucrose.

The underlying mechanism which accounts for the essentially straight-line relationship obtaining between the percentage of filtered water excreted and the percentage of filtered chloride excreted might possibly be explained by the presence of a non-reabsorbable agent or the excess of a reabsorbable substance such as glucose, which because of its osmotic value retains both water and salts in the proximal tubule, so that isosmoticity, normal to the proximal tubule (12), is maintained. The recent work of Wesson and Anslow (13) shows that during osmotic diuresis the osmotic pressure of the urine remains within limits of experimental error equal to that of plasma. If, then, an isosmotic condition is the result, a definite ratio between the retained water and salts in the tubule should follow. In the presence of relatively unchanging filtration rates and plasma concentrations of chloride, such a relationship should result in a urinary chloride concentration, which is essentially constant during the diuresis. The present results show that in all experiments the urinary chloride concentrations during diuresis remain within a rather limited range except at the beginning and end of the diuresis. The deviations from the relative constancy of urinary concentration at the lower urine flows might be explained by the greater effectiveness of distal tubular reabsorption of chloride or in some instances by changes in reabsorption of water. With greater flow, to cite Wesson, Anslow and Smith (14), "the quantity of proximal urine delivered to the distal tubule is so great that the specific operations of the latter, normally small in absolute magnitudes, are swamped in the flood, so to speak, and the composition of the urine must come to reflect closely the composition of the fluid delivered to the distal tubule by the proximal system."

The above-mentioned mechanism should result not only in increased excretion of chloride but also of other substances in the glomerular filtrate. Our preliminary studies showed that with all these agents the sodium excretion paralleled that of the chloride ion. However, in the case of sucrose diuresis, our urinary potassium values were variable and did not follow a definite pattern. The results of Rapoport and associates (9) showed that during osmotic diuresis in man the sodium loss in relation to urine flow followed the straight-line relationship as did the chloride, but that potassium and phosphate did not follow the same pattern. This suggests perhaps that even in the presence of osmotic diuresis the tubules are more active in handling potassium and phosphate.

Either depressing or elevating the serum level of chlorides did alter the ratio between the percentage of filtered chloride excreted and the percentage of filtered water excreted during osmotic diuresis. This means that while the amount of chloride excreted did increase proportionately to the urine flow even with low levels of plasma chloride, the total amount of chloride or sodium excreted for a given dose of sucrose might be as low as $\frac{1}{3}$ to $\frac{1}{10}$ of that excreted by the animal with normal serum chlorides and sodium (15). A similar shift in chloride-water excretion ratio resulting in the saving of chloride was observed in some experiments after administration of desoxycorticosterone. In view of our previous discussion the most likely explanation would be a change in reabsorption in the proximal tubule. While in our study serum chloride levels during any one experimental period remained at fairly constant levels it is possible that the chloride water excretion ratio might change from the straight-line

relationship depicted in figure 2 to any point in the range depicted by the lines in figure 5, if the injected osmotic agent were given in a manner to change appreciably the serum chloride levels. In fact recalculation of some of our preliminary experiments indicated that such occurred when the sucrose was given rapidly enough to reduce the serum chloride levels from 103 to 93 mEq/l. This situation may hold for the experiments of Wesson and Anslow (13) in which they report that sodium or chloride and water are not reabsorbed in a constant ratio as the diuresis induced by hypertonic mannitol solutions increases in magnitude. The serum chloride levels reported by these authors showed a wide variation in each experiment, in one dog for example changing from 123.5 to 88.1 mm/l. during the diuretic period.

In keeping with our original purpose namely whether or not it would be possible to predict the extent and nature of the electrolyte loss when osmotic agents are used to produce diuresis or dehydration, several general statements can be made. Since the work of Rapoport and associates (9, 16) seems to agree closely with our results perhaps they will apply both for man and dog. It would appear that the extent of the sodium and chloride loss is proportionate to the urine flow as long as the serum levels remain constant. This relationship is not altered appreciably by the type of diuretic used except possibly in the case of electrolytic osmotic agents although the total chloride loss will vary with the ability of that agent to produce a mild, moderate or severe diuresis. The ability of an osmotic agent to produce diuresis is perhaps a function in part of volume of distribution, mode of excretion and metabolic fate. Further, the ratio between chloride and water excreted varies as the serum chloride levels, so that a low serum level causes a low total chloride loss and a high serum level, a high chloride loss with similar rates of urine flow. Desoxycorticosterone may also decrease the chloride loss. Figure 2 suggests that the type of diuretic agent may also modify slightly the ratio between excreted chloride and water but we have no explanation as to the mechanism involved. It would seem that the effectiveness of an osmotic diuretic in promoting excretion of sodium and chloride depends primarily on the extent of the diuresis induced and secondarily on the serum levels and possibly on the type of diuretic agent used. From our unpublished data and from the work of Rapoport and associates (9) it would seem that these statements may not apply in the case of potassium and phosphate.

SUMMARY

Diuresis brought about by hypertonic solutions of glucose, sucrose sorbitol, and urea is associated with an increased urinary excretion of chloride ion, this increased excretion varying as the urine flow. When the percentage of filtered chloride excreted is plotted against the percentage of filtered water excreted an essentially straight-line relationship is obtained. The straight-line character of this relationship is unaffected by the type of osmotic agent used, by plasma chloride level or by pituitrin or desoxycorticosterone acetate. It was suggested that this phenomenon might be due to decreased reabsorption of chloride in the proximal tubule as a result of the increased tubular concentrations of glucose. Varying the plasma chloride levels altered the ratio between the percentage of filtered water excreted and the percentage of filtered chloride excreted so that low or high plasma values were

associated with corresponding changes in total urinary chloride excretion. The possible factors affecting this reabsorption of chloride are discussed.

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SIMULTANEOUS COMPARISON OF RENAL BLOOD FLOW AS MEASURED BY THE FICK PRINCIPLE AND BY THE BUBBLE FLOW METER¹

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IT HAS been generally recognized (1, 2) that the clearance method of measuring renal blood flow using para-aminohippurate (PAH) or diodrast is unsatisfactory in abnormal states in which renal extraction is significantly decreased (3, 4). Without access to renal venous blood, the resulting inability to measure the renal arterio-venous PAH difference (A-R) directly makes it impossible in these abnormal conditions to assess how much of the decrease in clearance values is due to actual decreases in flow and how much is due to decreased tubular extraction. Yet it is in these very conditions that the recording of accurate flow measurements is most desirable. Thus when the advent of renal vein catheterization made possible clinical determination of A-R differences the Fick method based on the renal A-V PAH difference was seized upon as a means by which renal blood flow could be measured (theoretically at least) as long as the kidney is capable of excreting any measurable amount of urine and test substance. Certain basic assumptions, necessary to the application of the Fick equation, using PAH, to the renal vascular bed, have been reviewed by Phillips *et al.* (5). Additional ones not considered assume that PAH is not destroyed by the kidney, that renal lymphatic flow is negligible (6), and that there are few or no anastomoses with an extraction ratio different from the kidney as a whole by which blood can leave the kidney by routes other than via the renal vein (7). Furthermore, a straightforward experimental comparison of the Fick principle against a direct method of renal blood flow measurement has never been accomplished, although comparison can be made by 1) calculations derived from Selkurt's data (4), and 2) a compilation of these data by a comparison of direct flows from Selkurt's tube against PAH clearance flows, and that of Phillips *et al.*, who compared PAH clearance flows with PAH Fick flows (5). The first derivation is not valid, however, because no consideration was given to the diffusion of PAH from red cells to plasma in the renal venous samples. Calculation of the data comprising the second derivation is at best only an approximation.

The purpose of these experiments was to make such a direct comparison between measuring renal blood flow by the Fick method and by a direct method with the use of the bubble flow meter. The bubble flow meter, the virtues and shortcomings of which are discussed elsewhere (8), has advantages over the tube used by Selkurt in

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TABLE 1. COMPARISON OF RENAL BLOOD FLOW AS MEASURED BY THE FICK PRINCIPLE AND BY THE BUBBLE FLOW METER

EXPER. NO. ¹ AND DATE	WT.	URINE RATE, CC/MIN/LEFT KIDNEY	URINE VOL.	P-AMINOHIPPURATE DYNAMICS			BUBBLE FLOW METER WHOLE BLOOD CC/MIN/LEFT KIDNEY	BF _{PAH} BF _{BFM}
				PAH levels (corrected) whole blood		Urine Vol./A-R CC/MIN/LEFT KIDNEY		
				arterial	venous			
	kg.		mg/min.	mg. %	mg. %			
I 1/ 4/49	II	3.75	13.14	62.0	54.7	180	149	1.21
	II	3.00	11.34	53.6	46.3	155	136	1.14
I 1/ 4/49	II	3.75	13.14	62.0	54.7	180	149	1.21
		3.00	11.34	53.6	46.3	155	136	1.14
2 1/11/49	13	2.30	14.01	16.8	10.5	222	199	1.12
		2.10	12.27	18.7	12.3	192	196	0.98
		2.10	11.73	17.7	11.2	180	157	1.15
3 1/17/49	14	1.40	12.24	33.1	28.0	240	224	1.07
		1.12	10.32	27.4	22.2	200	190	1.05
		0.70	7.04	22.9	18.0	144	150	0.96
4 1/28/49	21	1.25	17.78	16.1	9.1	254	257	0.99
		1.15	15.06	14.6	7.9	225	239	0.94
		1.40	15.54	12.4	5.9	239	233	1.03
		1.23	12.16	10.6	4.1	187	198	0.94
5 2/ 1/49	18	1.88	11.62	32.9	25.2	151	148	1.02
		1.53	10.56	28.0	19.7	127	126	1.01
6 2/12/49	14.8	3.20	16.61	38.9	31.5	224	218	1.03
		3.30	17.09	45.3	37.4	216	209	1.03
		3.00	15.72	45.6	37.1	185	174	1.06
7 2/23/49	15.5	2.20	19.15	46.7	36.3	184	175	1.05
		1.15	17.71	69.9	58.6	157	152	1.03
8 3/ 1/49	14.3	1.14	13.34	57.6	50.6	190	171	1.11
		1.24	12.98	58.3	51.1	180	168	1.07
9 3/ 9/49	19	1.18	16.48	16.4	7.7	189	203	0.93
		1.58	17.49	17.3	9.1	213	208	1.02
		1.83	16.47	19.9	10.2	170	178	0.96

Statistical analysis of RBF_{PAH}/RBF_{BFM} (See RESULTS): Mean = +0.025; S.D. = 0.059; S.E. of the mean = 0.013; *t* value = 1.9

¹ Experiment 1 was on a female dog; experiments 2-9 were on male dogs.

measurement of direct renal venous outflow in that it permits numerous measurements at short intervals and requires no withdrawal of blood during measurements.

In these experiments the A-R difference of PAH was determined on whole blood rather than plasma, thus avoiding the need for immediate centrifugation or for

application of a constant time correction factor, the exactness of which may be open to question.

METHODS

Operative Procedure. Male dogs and one female dog were used ranging from 11 to 21 kg. Anesthesia was produced by 1.5 gm. of 20 per cent urethane/kg. by stomach tube and 60 to 80 mg. of one per cent chloralose/kg. intravenously. An endotracheal tube was inserted. In order to obtain unilateral urine collection, the left ureter was exposed through a left paramedial lower abdominal incision (subsequently closed) and a polyethylene catheter inserted so that the tip just reached the renal pelvis. Diuresis was maintained by a slow, continuous intravenous drip of 20 per cent mannitol. Arterial samples were collected from the femoral artery. In order to measure directly the venous outflow and to obtain renal venous samples the renal vein was exposed from a flank incision by careful dissection of the perirenal adipose tissue. The left spermatic vein was ligated near its entrance into the renal vein. The right external jugular vein was also exposed.

Beginning approximately $2\frac{1}{2}$ hours before the initial clearance period 6 gm. of 20 per cent PAH were given intravenously. At hourly intervals this same dosage was repeated in order to maintain high constant or slowly falling blood levels. When these preliminary preparations were completed a siliconed bubble meter with an internal diameter of 4.5 mm. was installed between the renal vein and the right external jugular vein. The meter had previously been filled with saline containing 3 cc. of heparin. This procedure resulted in renal ischemia which was deliberately varied from 2 to as much as 14 minutes.

Previous to the experiments the bubble meter volume was calibrated dynamically at varying flows by the use of a perfusion pump with blood as the fluid medium.

After the meter was inserted the wounds were closed and in some experiments the animals transfused with 90 to 120 cc. of whole dog blood, containing a PAH concentration roughly equivalent to that in the dog. When the readings of the bubble meter indicated maximal flow and relative stability, clearance periods were begun, each of 20 minutes duration. The average elapsed time from the start of the experiment to the first clearance period was $2\frac{1}{2}$ to 3 hours. At the beginning of each period simultaneous blood samples from the femoral artery and renal vein were drawn into oiled syringes and transferred to heparinized tubes. A lag time of 2 minutes was used in the urine collection. Two to 4 clearance periods were taken on each animal. During the periods bubble flow meter readings to the nearest 0.05 second were taken once or twice per minute. To prevent the formation of fibrin in the bubble meter 0.5 cc. of heparin was given intravenously every half-hour. At the end of the experiment both kidneys were weighed and examined.

Chemical Analyses. All chemical analyses were carried out in duplicate. Determinations of PAH were carried out on whole blood and urine by the method of Bratton and Marshall as modified by Smith. Preliminary experiments showed that 1-cc. pipettes delivered only 0.96 ± 0.01 cc. of whole blood at a room temperature of 25°C. and that whole blood recoveries averaged 0.93 cc. However, if the pipette washings of one cc. of physiological saline were included in the recovery sample, the recovery in 3 preliminary and the 9 reported experiments ranged from 0.95 to 1.02 cc. with an average of 0.98, S. D. of 0.02 and S. E. of the mean of 0.006. Because the variability between animals was only slightly more than that within each animal all arterial and venous PAH whole-blood concentrations were corrected for 0.98 recovery. All photoelectric readings were made on the Evelyn colorimeter. PAH concentrations were read from a standard curve.

Calculations. The following formulas were used in the calculations of renal blood flow (cc./minute):

$$A. \quad \text{Bubble Meter (RBF}_{\text{FM}}) = \left(\frac{t}{60} \cdot V^1 \right) + V$$

$$B. \quad \text{Fick PAH (RBF}_{\text{PAH}}) = \frac{UV}{A_B - R_B}$$

where

- t = average of the times (in seconds) for the bubble to pass through the meter,
 V = urine volume in cc/minute,
 V^1 = dynamic volume of the bubble meter at the flow under consideration
 UV = urine, PAH
 A_B = arterial whole blood, PAH
 R_B = renal venous whole blood, PAH.

RESULTS

There were 9 experiments involving a total of 24 clearance periods. The renal blood flow measured by the direct method varied from 126 to 257 cc. of whole blood/minute/kidney, while the flows measured by the Fick method varied from 126 to 260. The direct flow figures include the addition of the minute volume of urine to the flow as measured by the bubble meter. The ratio of the whole blood flows as determined by PAH and bubble meter, respectively, ranged from 0.93 to 1.21. In 17 of 24 periods the ratio was greater than one. The difference of this ratio from unity has been analyzed statistically. The difference of the mean ratio of RBF_{PAH}/RBF_{BFM} is $+0.04$, S. D. ± 0.072 , and S. E. of the mean ± 0.015 . This $+0.04$ difference is statistically significant at the 5 per cent level, the t value being 2.15. However, this significance is entirely dependent upon inclusion of the widely divergent first experiment. With the omission of the first experiment, analysis of the remaining 22 periods shows a mean difference of $+0.025$, S.D. of 0.059, and S. E. of the mean of 0.013. This difference is found to have no statistical significance at the 5 per cent level, the t value being 1.9. The latter values are obviously more representative of the entire series and are therefore referred to hereafter.

DISCUSSION

Comparison of the flows as measured by the two methods resulted in a surprisingly close agreement, the mean difference, standard deviation and standard error of the mean being quite small in view of the numerous apparent sources of random error. However the question still arises as to whether or not one may ascribe this small mean difference to a systematic error or an actual cause. No systematic error could be identified. There remains the possibility that blood entered the kidney and left by a channel other than through the renal vein. Only two avenues of escape seem possible: 1) via the renal lymphatic channels and 2) via capsular or other vascular anastomoses. Unfortunately, as a result of such escape, either of the flow measurement methods or both might theoretically be sources of error depending upon the concentration of PAH in the escaping blood or lymph as opposed to that in the renal vein. Thus to draw any conclusion regarding flow through these channels becomes more difficult, yet the results would seem to indicate that they are not outlets for appreciable amounts of fluid, although they may have accounted for the 2.5 per cent difference.

Certain also apparent deviations from the physiological state in these animals included 1) anesthesia, 2) surgical trauma, 3) presence of the bubble meter in the renal venous circuit, 4) blood loss and 5) renal ischemia. The effect of the bubble meter on the pressure in the renal vein was measured in several animals; no measurable rise was ever seen. Although the other effects of these factors could not be

individually quantitated it was shown by concurrent studies⁴ not herein reported that renal function was within the normal range during nearly all the periods except those in which previous renal ischemia was deliberately prolonged. In those animals creatinine clearances as low as 6 cc/minute and PAH Tm's as low as 3 mg/minute were recorded. Thus by prolonging the ischemia not only were the renal flows varied over a wider range, but of more importance flow comparisons were made in some periods during which renal function was so decreased that presumably clearance flows would have been quite inaccurate. The fact that even under these rather severe conditions, Fick blood flows compare just as favorably with values obtained by direct measurements as in those animals with essentially normal kidneys, indicates that renal abnormalities capable of rendering straight clearance measurements of flow meaningless are unlikely to alter the validity of the Fick principle as applied to renal blood flow measurements.

SUMMARY

Renal blood flow in the dog measured by the Fick principle using PAH was compared with the flow measured directly by the bubble flow meter. The renal blood flow_{PAH}/Renal Blood Flow_{BFM} ratio was 1.025. The mean difference was not statistically significant.

The authors wish to express their gratitude to Dr. Carl F. Schmidt and to Dr. J. Kapp Clark for their advice and encouragement during the course of these studies.

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⁴ Mean arterial blood pressure, creatinine clearances, PAH Tm's and filtration fractions. In addition all kidneys were given a post-mortem examination. In every instance the one operated upon was hyperemic and edematous to some extent and weighed more (up to 15%) than its fellow. The degree of change was roughly parallel to the duration of renal ischemia.

REFLEX CARDIAC INHIBITION IN THE GANOID *ACIPENSER STURIO*¹

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THE present investigations were made on the rare occasion of having a living sturgeon (*Acipenser sturio*) in good condition at disposal. Up to now, no representative of the group of ganoids has been included in such experiments. Furthermore, no electrographic methods have been used for recording the heart functions under these conditions.

McWilliam (1) was the first to study the reflex excitation of the cardiac nerves of fish in a classic paper on the structure and rhythm of the heart in fishes (1885). He found that the most different stimuli produced a standstill of the heart in the eel, carp, perch, rudd and other fishes. McWilliam mentioned the gill apertures, the gills, the internal surface of the branchial chamber, the skin of the head and certain portions of the cutaneous surface as places from whence these reflexes can easily be provoked. He found the caudal fin and the skin of the tail in eels, and the parietal peritoneum especially sensitive. Stimulation of the mucous membrane of the mouth and the pharynx and the central end of the cut vagus was effective, and so was a weak stimulation of the optic lobes.

The next to devote a thorough study to these problems was Schoenlein (2). He found similar reflexes in the group of selachians investigated. He proved that Atropin prevents the heart's standstill due to these reflexes but not the respiratory standstill. After Schoenlein, different authors corroborated the findings of McWilliam and Schoenlein on bony fishes, and on selachians. Among them, Lutz (3) devoted different papers to special investigations in this field, and so did von Skramlik (4), both citing most of the pertinent literature.

METHOD AND RESULTS

All unphysiological kind of stimuli were eliminated, such as pinching with a forceps or electric stimulation. Only gentle touching with the finger or the dull part of a metallic instrument, where the finger seemed too big to touch certain spots, was used. The animal was 57 cm. in length. It was attached to a fish board, a small tube was inserted into the mouth, and seawater kept running from there over the gills. No narcosis was used. The heart function was registered electrographically with direct leads. The exploring electrode was put on the exposed heart. A modified Wilson Central terminal was used as an indifferent electrode, as previously described (5, 6). The exposure of the heart was performed without any bleeding. The duration of the used stimuli was marked on the electrocardiogram.

The heart rate at the beginning of the experiment was approximately 70/minute. The electrocardiogram does not differ essentially from that taken in other fishes (5, 6).

The ganoid *Acipenser* reacted like teleosts and selachians with a standstill of the entire heart when stimulated by touching the aperture of the mouth, the mucous

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membrane of the palate or any other part of the mouth cavity, pharynx or gills. A standstill of the heart could be produced by touching an eyeball with the finger or by touching any of the 4 barbels, the skin of the abdomen or the fins.

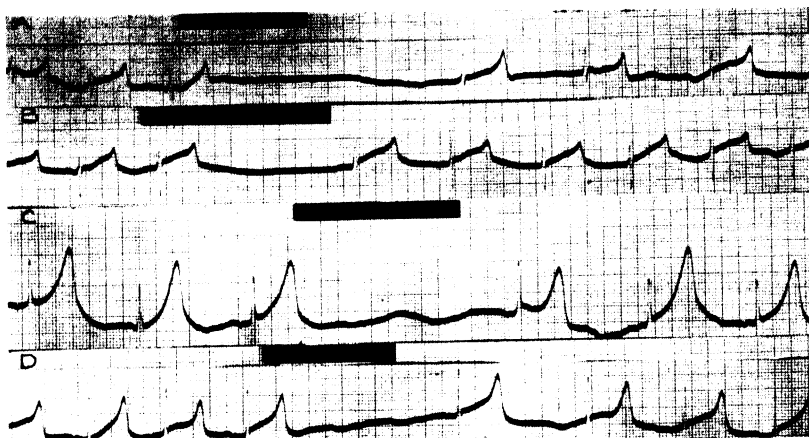


Fig. 1. DIRECT LEADS taken from different points of the sturgeon's heart. The mark indicates the time of stimulation. A: lip pressed with fingers. B: palate touched with dull end of forceps. C: rostrum gently touched with finger. D: right eyeball touched with finger. One small square = 0.04 sec.



Fig. 2. SAME STURGEON. A: head behind rostrum gently touched with finger. B: right gill touched with forceps. C: tail strongly pressed. One small square = 0.04 sec.

In all these cases, if the stimulus was of short duration, the standstill of the heart lasted somewhat longer than the stimulus (see figs. 1 and 2). When sinus rhythm started again it was at first slower than before.

If the stimulus lasted very long, the reflex became exhausted. Sometimes the auricle was beating, but not the ventricle (a-v block, dropped beats) or escaped

ventricular beats appeared, similar to nodal beats or a nodal rhythm, and finally the heart was beating again regularly, but with a slower rhythm at first. Proof of this is that the heart was beating with the tube in the mouth, which unquestionably presented a continuous stimulus, but did stop beating for a time only when the tube was first inserted. Afterwards, when this lasting stimulus was no longer able to stop the heart beat, a gentle touching of the palate next to the tube with the dull side of a forceps still did it promptly (see fig. 1). Simultaneously, with the stopping of the heart beat, a reflex inhibition of the respiratory movement took place, but both these were not exactly related. In protracted stimuli the respiration, as a rule, stopped much longer than the heart beat.

The sturgeon's skin is armed with 5 rows of bucklers and the entire skin is rough with small irregular plates, the head especially being covered with bony plates. The experiment proved (see figs. 1 and 2) that the skin, especially of the head of the sturgeon, is extremely sensitive. The slightest touch with the finger of any part of the head or rostrum immediately stopped the heartbeat and the respiration.

In contradistinction to the observations of McWilliam on eels, the sensitivity of the skin and fins of the sturgeon decreases from the head towards the tail. Slight touching of the tail may have no ostensible reflex effects, and even a strong pressure of the tail with the fist of the experimenter produced no standstill, only a marked slowing of the heart rate, in which case reflexes from parts of the skin nearer to the head produced by movements of the animal may have been a factor (fig. 2).

In confirmation of McWilliam's experiments, in the eel (*Anguilla bosteniensis*) the tail was found to be one of the most sensitive parts of the animal's body. Not only heart standstills due to gentle touching of the tail were observed, but it was observed repeatedly that if, for other purposes, the tip of the eel's tail was cut with a razor blade, the animal was paralyzed and remained so for a long time, even though no significant loss of blood occurred. Returned to the seawater basin, it remained lying on its back for a long time, not breathing, or else breathing very weakly. That happened so promptly each time this experiment was tried, that it can be recommended as a lecture or course experiment, to demonstrate the effect of such an insignificant stimulus to a big animal via reflexes.

DISCUSSION

The fact that the ganoid *Acipenser* behaves, as far as the inhibitory reflexes on heart and respiration are concerned, similarly to other fishes, is not astonishing. One question that was scarcely discussed in all the previous papers on reflex inhibition of the heart and of the respiration in fishes is that of what role this reflex may play in the normal physiology of fishes. It seems beyond doubt that if a slight touch of the head of the sturgeon produces a longlasting standstill of the heartbeat and respiration, similar reflexes must have an influence on the normal behavior of the animal in his normal surroundings. That means that not only touching some solid material with the snout and with the head may produce a reflex reaction of the fish, but less strong stimuli such as a strong current within the water produced by an approaching bigger animal, or by a reflection of the water from a strong obstacle, may also be perceived by the fish through its extremely sensitive organ within the skin of the head.

This may hold for other fishes as well, and may explain to a certain degree the familiar observation that an entire shoal of fish in the water suddenly turn altogether and swim in another direction.

It may also be of a definite meaning concerning the behavior of fish, that in some species, like the sturgeon, the sensitivity of the skin decreases from the head to the tail, and in other species, like the eel, the tip of the tail proves to be the most sensitive part of the surface of the animal.

SUMMARY

In the ganoid *Acipenser* reflexes inhibiting the heart beat and the respiration can be provoked as in other fishes (teleosts and Selachians). The sensitivity of the skin of the sturgeon concerning these reflexes decreases from the head to the tail. The inhibitory reflexes were studied electrocardiographically. A longlasting paralyzing effect of cutting the tail of eels is described. The physiological meaning of this kind of reflexes in fishes is discussed.

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EFFECT OF NORMAL AND ABNORMAL CHANGES OF INTRATHORACIC PRESSURE ON EFFECTIVE RIGHT AND LEFT ATRIAL PRESSURES¹

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RECENT work by our group has interrelated right and left atrial hemodynamics in open-chest dogs and applied the knowledge thus obtained to the problem of atrial hemodynamics in the presence of experimentally produced inter-atrial communications (1, 2). However, we wish to be the first to criticize our experiments and to point out that, although the dynamic factors elucidated heretofore are fundamental, they are subject to modification by external forces not normally operative in open-chest experiments. The chief physiological modifying factor is changes in intrathoracic pressure due to respiration. We are therefore extending our studies of the hemodynamics of inter-atrial septal defects to the closed-chest dog.

Since left atrial pressure has been studied so little, it has been necessary first of all to establish the effect of variations of intrathoracic pressure on right and left effective pressures simultaneously recorded from normal hearts. This paper, therefore, deals with the relation between right and left effective atrial pressures during normal respiration and in states in which there are abnormally great fluctuations of intrathoracic pressure.

METHODS

Medium-sized mongrel dogs anesthetized with morphine and sodium pentobarbital were used in this study. The thorax was opened by a right intercostal incision in the fourth interspace after inserting a tracheal cannula and preparation of a carotid artery and the right external jugular vein for cannulation. Mild but adequate artificial respiration was maintained while the thorax was open. A rigid cannula was introduced into the left atrium through the pulmonary vein of the right middle lobe of the lung. The cannula was fixed in the optimum position by means of clamps and the thorax closed by layers around the cannula with the aid of the vaseline and gauze technique and the lungs reinflated. No leakage could be detected. The right atrium was entered via a sound passed through the jugular vein and the aorta similarly by way of a carotid artery. Pressure pulses were recorded optically from the aorta and atria by Gregg manometers of appropriate sensitivity and adequate frequency. In some experiments aortic pressures were recorded by a Sanborn electromanometer connected to a large Hindle galvanometer.

Intrathoracic pressures were recorded from both right and left sides of the thorax by means of trocars placed in the immediate vicinity of the atria. The trocars were introduced via stab wounds and the pressures recorded by an air transmission system and Frank segment capsules. It seemed advisable to measure intrathoracic pressures on both sides since a uniform pressure does not exist in all parts of the intact thorax (3, 4). However, after pneumothorax and subsequent closure only slight and consistent differences were noted—usually less than 10 mm. saline.

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The atrial and intrathoracic pressure beams were calibrated against a single limb saline manometer whose zero pressure was set equal to the level of the animal board. All pressures are therefore relative. A calibration of all beams was made immediately after taking each record.

After closing the thorax and obtaining satisfactory atrial pressure tracings numerous records, carefully calibrated, were taken during quiet, spontaneous respiration. Frequently, rate and/or depth would change during this period of observation, making it possible to assess the effect of such changes. These observations on the effect of changing intrathoracic pressure were carried out before introducing procedures such as infusions and nerve stimulations which might conceivably alter the normal pressure relations. A representative record is reproduced in figure 1 which shows the usual configuration of the pressure pulse contours and the arrangement of the recording beams.

Abnormal fluctuations, i.e. increase or decrease of intrathoracic pressure above or below the pressure limits of spontaneous respiration, were produced by various manipulations. A profound decrease of intrathoracic pressure was produced by occluding the tracheal cannula during expiration and allowing an inspiratory movement to be made (Müller's experiment). Producing an increase of intrathoracic pressure in the anesthetized dog physiologically similar to the Valsalva experiment in man presented problems which were not entirely solved, an experience shared by others (5). Two methods of increasing pressure were utilized: 1) abdominal compression with the tracheal cannula occluded, and 2) stimulation of the central end of the divided sciatic nerve with the trachea occluded.

Effective atrial pressure was calculated in the usual way, by algebraic difference between the recorded atrial pressure and the coexisting intrathoracic pressure. For convenience in surveying the records, effective atrial pressures were calculated at a point on each cardiac cycle just before the second heart sound artifact (the U point, see marking on atrial cycle, fig. 1) during two or more consecutive respiratory cycles. As emphasized in a preceding paper (2), this instantaneous pressure value was chosen because it represents a significant and consistently measurable point on the atrial pressure curve just in advance of ventricular filling. A more detailed analysis of the variation in effective atrial pressures during each cardiac cycle was made by measuring the records 10 points to the inch (paper speed, one inch = 1.00-2.00 milliseconds) throughout a single typical respiratory cycle, a procedure necessitating many thousands of measurements and calculations. From these calculations it was possible to construct effective right and left atrial pressure curves showing the pressure at any time during each heart beat in the respiratory cycle.

RESULTS

Effective Atrial Pressures During the Expiratory Pause. Effective atrial pressures were calculated at the U point near the end of the expiratory pause in 8 experiments. In all experiments effective left atrial pressure (EP_{LA}) exceeded effective right atrial pressure (EP_{RA}). The difference between EP_{LA} and EP_{RA} ranged from 21 to 114 mm. saline. Closer analysis showed that in one-half of the experiments EP_{LA} exceeded EP_{RA} throughout each cardiac cycle, whereas in the remainder of the experiments EP_{RA} exceeded EP_{LA} for a brief time (0.02-0.1 seconds) during some phase of the cycle, usually near the beginning or peak of right atrial systole. These observations are similar to those made on open-chest dogs by Opdyke *et al.* (1).

Effect of Normal Intrathoracic Pressure Changes on Atrial Pressures. Plot A, figure 2, illustrates the usual changes in the effective atrial pressures observed during normal quiet respiration. Intrathoracic pressure decrease during inspiration in the various experiments ranged from 30 to 58 mm. saline and the respiratory rate varied from 6 to 20 cycles, minute.

With the onset of inspiration a consistent increase of EP_{RA} was observed. The increment of pressure, however, was not large, ranging from 20 to 30 mm. saline. During the passive expiration phase EP_{RA} decreased rather abruptly, usually to a level near that existing just before inspiration began. Occasionally, at the end of

passive expiration EP_{RA} decreased to less than the pre-inspiratory pressure, in which case EP_{RA} increased slightly during the expiratory pause. Slower and deeper respiratory movements exaggerated the changes observed.

In contrast to the consistent change observed in EP_{RA} , the EP_{LA} exhibited only slight change with an inspiratory decrease of intrathoracic pressure. The direction of change, when such occurred, was not consistent either from beat to beat or from experiment to experiment. In any event, the change, amounting to only a few millimeters of saline, was too small to be significant. Faster respiratory rates and shallower depth tended to reduce the slight variation still further.

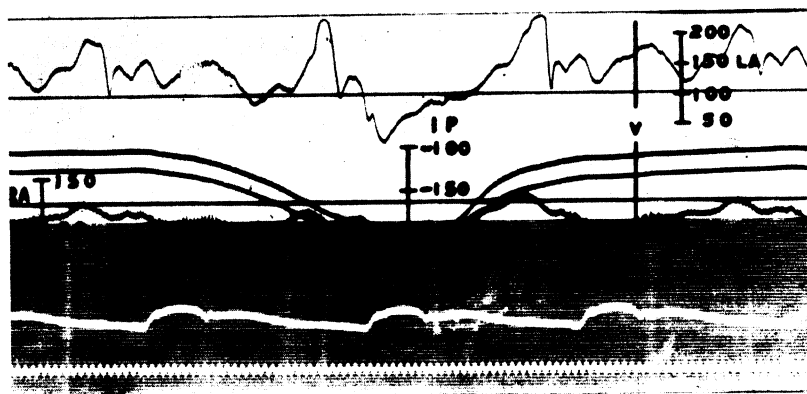


Fig. 1. CHANGES OF LEFT ATRIAL PRESSURE (top curve), right and left intrathoracic pressures (parallel curves, middle), right atrial pressure (fourth from top), and aortic pressure (bottom) during an inspiratory attempt with trachea occluded (Müller's experiment). Calibrations in millimeters of saline, except aortic (140/102 mm. Hg).

In summary, decrease of intrathoracic pressure due to inspiration of normal depth and duration increases effective right atrial pressure moderately, but has no significant effect on that of the left atrium.

Effect of Greatly Reducing Intrathoracic Pressure. Greater reduction of intrathoracic pressure was accomplished by performing Müller's experiment. Intrathoracic pressure was lowered anywhere from 90 to 160 mm. saline by allowing an inspiratory movement to be made with the tracheal cannula occluded. The occlusion was not maintained for more than 3 or 4 consecutive respiratory attempts. Figure 1 and figure 2, plot B, are typical examples of the pressure pulses and results obtained from this type of experiment. EP_{RA} markedly and consistently increased immediately upon the onset of decreasing intrathoracic pressure, continued to rise until the inspiratory movement was at least two-thirds complete, and usually continued to rise up to the point of lowest intrathoracic pressure. In figure 2, plot B, the pressure rise was equal to 49 mm. saline. With the beginning of relaxation of inspiratory muscles EP_{RA} decreased promptly, descending to or going below its pre-inspiratory level by the time intrathoracic pressure reached the apneic level. During the early part of the period of apnea EP_{RA} increased to its pre-inspiratory pressure (if it had

decreased below this level when intrathoracic pressure was restored to the apneic level). EP_{LA} also behaved in a predictable manner during Müller's experiment. After a short lag it increased as intrathoracic pressure decreased. The increase usually began after the inspiratory movement was one-third completed. The total pressure increment (about 20 mm. saline in fig. 2, *plot B*) was always less than the

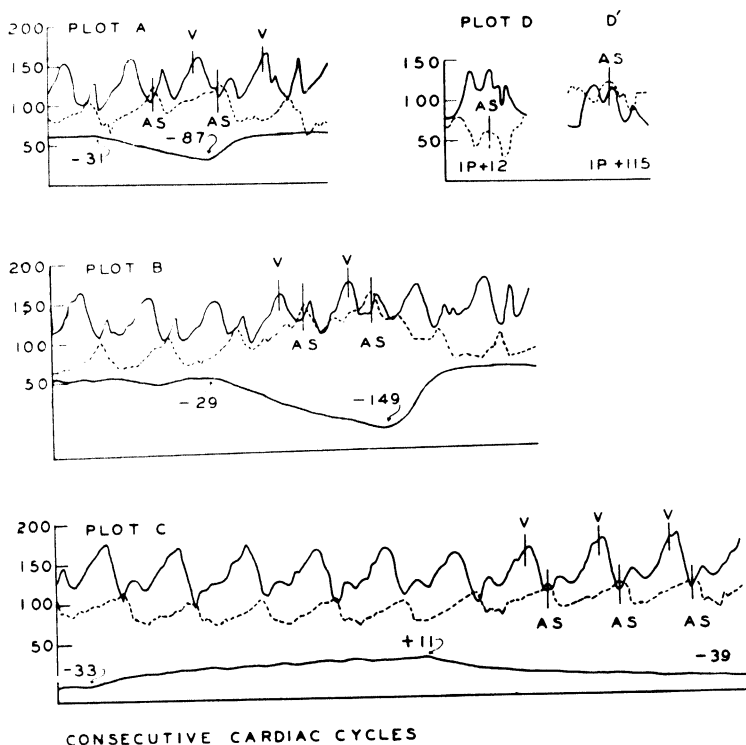


Fig. 2. EFFECTIVE LEFT ATRIAL PRESSURE (top, solid line), effective right atrial pressure (broken line), and intrathoracic pressure (bottom, solid line) during normal respiration (*plot A*), Müller's experiment (*plot B*), and positive pressure lung inflation (*plot C*). *Plots D* and *D'* represent selected cardiac cycles before and during a maintained active expiratory attempt produced by sciatic stimulation. Ordinates are in millimeters of saline. The maxima and minima of intrathoracic pressure (mm. saline) during each respiratory cycle are indicated directly on the plot. (See text.)

comparable increase in EP_{RA} . However, EP_{LA} did not decrease as did EP_{RA} when the inspiratory muscles were relaxed, but continued at its elevated level well into the period of apnea (expiratory pause). EP_{LA} declined to the pre-inspiratory level sometime during the apneic period.

In summary, greatly decreased intrathoracic pressure results in an increase of both EP_{RA} and EP_{LA} , but the former increases more promptly and to a greater extent than the latter. With the restoration of the apneic level of intrathoracic

pressure EP_{RA} returned quickly to its former level, whereas EP_{LA} exhibits a considerable lag in doing so.

Effect of Increased Intrathoracic Pressure on Effective Atrial Pressures. Many situations exist in which an increase of intrathoracic pressure is an important feature. One need only mention such instances as coughing, sneezing, straining and artificial respiration by positive pressure lung inflation. It was of importance, therefore, to study the effect of abnormally increased intrathoracic pressure on effective right and left atrial pressures.

As a first approach to this problem, records of atrial pressures were obtained while the dogs were subjected to mild positive pressure lung inflation with passive expiration through an open side tube. The respirator was so constructed that the inflation and deflation periods were of equal duration. The maximum intrathoracic pressure increase during inflation ranged from 34 to 60 mm. saline in the various experiments.

The effective atrial pressure variations during an artificial respiratory cycle of a typical experiment are shown on figure 2, *plot C*. Although the pressure changes were not great, it can be seen that both EP_{RA} and EP_{LA} declined as intrathoracic pressure increased. The maximum pressure change during inflation shown on *plot C* was about 12 mm. saline for both effective pressures. This small decrease in pressure was consistently seen in all experiments. In some experiments EP_{RA} leveled off before inflation was complete and EP_{LA} continued to decrease well into the period of lung deflation. However, both effective pressures usually increased with the onset of deflation (i.e. returned to normal).

Since it was evident that only minor variations in effective atrial pressures resulted from mild positive pressure lung inflation, other methods of increasing intrathoracic pressure were tried.

It was possible to increase intrathoracic pressure by as much as 120 mm. saline by gentle abdominal compression with the trachea occluded. The effect of an intrathoracic pressure increase produced in this fashion resulted in rather variable patterns of change in right and left effective atrial pressures. Analysis of records from 3 different experiments gave the following results: In one experiment both EP_{RA} and EP_{LA} decreased 12 mm. saline, but initially $EP_{LA} > EP_{RA}$; in a second experiment both EP_{RA} and EP_{LA} increased 15 mm. saline, but $EP_{LA} > EP_{RA}$; however, in another record from the same experiment EP_{LA} increased 15 mm. saline, while EP_{RA} decreased 13 mm., EP_{LA} still being greater than EP_{RA} ; in a third experiment EP_{LA} increased 11 mm. while EP_{RA} decreased 31 mm. saline, $EP_{LA} > EP_{RA}$. All of these pressures were calculated at the *V* point. It should be emphasized that in all of these instances EP_{LA} was initially greater than EP_{RA} and, despite the unpredictable variation found, this relationship was never altered.

Sciatic stimulation with the trachea occluded usually resulted in deep inspiratory and active expiratory movements at a rapid rate. Intrathoracic pressure at the end of the inspiratory movement therefore decreased below the level reached at the end of a normal inspiration and attained a higher level than during the apneic phase of normal respiration. In one experiment these extremes were -117 mm. and $+150$ mm. saline, and in another -100 and $+178$ mm. saline. Atrial pressures as recorded

also showed extreme fluctuations, in fact so much so that careful judgment was required to differentiate whether the deformed pressure pulses resulted from the rapid change of intrathoracic pressure or whether the movements of the thoracic cage caused dislocation of the cannulae and subsequent artifacts on the record. However, sufficient data were obtained to indicate the probable state of affairs.

Table 1 contains data from a typical response to central sciatic stimulation. Only pressures at the V point are presented, but these are reliable indices as to the behavior of the pressures at other points during the atrial cycle.

TABLE 1. ATRIAL PRESSURES MEASURED AT THE V POINT
BEFORE AND DURING CENTRAL SCIATIC STIMULATION

RESP. CYCLE BEFORE OR AFTER BEGINNING STIMULATION	RECORDED ATRIAL PRESSURE		INTRATHORACIC PRESSURE	EFFECTIVE ATRIAL PRESSURE		EP _{LA} - EP _{RA}
	Right	Left		EP _{RA}	EP _{LA}	
	mm. saline	mm. saline		mm. saline	mm. saline	mm. saline
<i>Measured at or near end of inspiration during normal respiration</i>						
3rd before	50	140	-45	95	185	+90
2nd "	54	140	-40	94	180	+86
1st "	36	120	-75	111	195	+84
<i>Measured at or near end of inspiratory movement during central sciatic stimulation</i>						
2nd after	57	135	-35	92	170	+78
4th "	58	145	-42	100	187	+87
6th "	56	146	-85	141	231	+90
7th "	28	80	-110	138	190	+52
<i>Measured near end of apneic phase of normal respiration</i>						
3rd before	65	157	-7	72	164	+92
2nd "	65	152	-10	75	162	+87
1st "	65	155	-18	83	173	+90
<i>Measured at or near end of forced expiratory movement during stimulation</i>						
1st after	105	210	+57	48	153	+105
3rd "	125	230	+72	53	158	+105
5th "	160	285	+137	23	148	+125
7th "	175	290	+150	25	140	+115

When progressively greater inspiratory movements (greater negative intrathoracic pressure) resulted from nerve stimulation, it will be noted that both EP_{RA} and EP_{LA} also increased progressively for a number of respiratory cycles (to 6th cycle after beginning of sciatic stimulation, see table 1). The differences between EP_{LA} and EP_{RA} indicate that perhaps EP_{LA} is increased somewhat more than EP_{RA} by the greater decrease of intrathoracic pressure. Whether or not the decrease of effective pressures during the seventh respiratory cycle is significant is not known. Active expiration causes a progressive decrease of both EP_{RA} and EP_{LA}, the latter somewhat less than the former. Since the decrease in effective pressure is greater on the right side, the difference (EP_{LA} - EP_{RA}) becomes slightly greater. As in the case of abdominal compression, EP_{RA} never exceeded EP_{LA} at any time.

Another experiment involving sciatic stimulation and illustrated in figure 2,

plot D, is of particular interest since a maintained forced expiratory movement was obtained. Mild stimulation of the central end of the sciatic nerve with the tracheal cannula occluded resulted in a progressive increase of intrathoracic pressure. The progressive recruitment of expiratory muscles could be seen as the stimulation continued. Intrathoracic pressure increased from an apneic level of 12 mm. saline to 115 mm. The dog made no inspiratory attempt for about 5 seconds after the maxi-

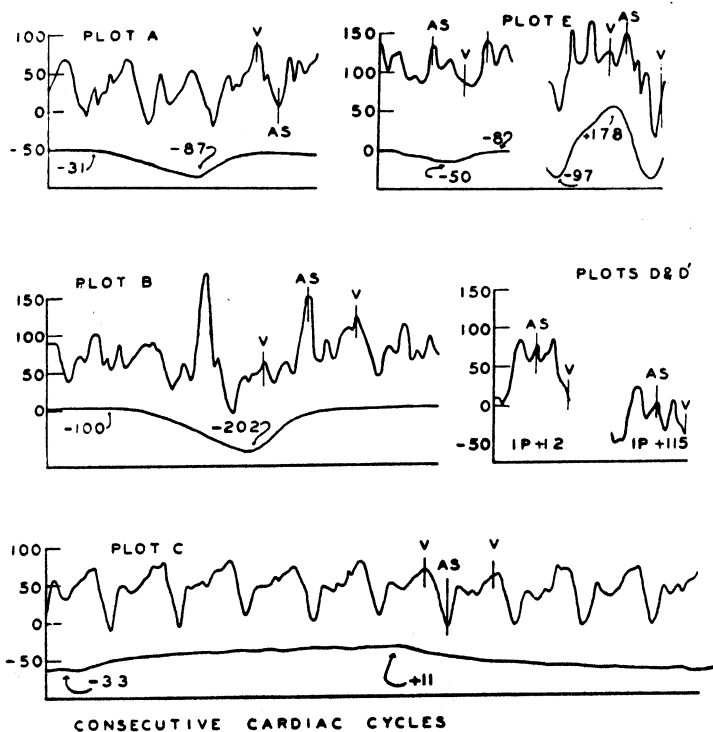


Fig. 3. INTER-ATRIAL PRESSURE GRADIENTS (differential pressure) during a normal respiratory cycle (plot A), Müller's experiment (plot B), and positive pressure lung inflation (plot C). Plots D and D' correspond to plots D and D', figure 2. Plot E shows effect of violent respiratory movements produced by sciatic stimulation. Ordinates are in millimeters of saline. Maxima and minima of intrathoracic pressure are indicated directly on plot. (See text.)

mum intrathoracic pressure was reached. Figure 2, plot D, shows that as a result of the increase of intrathoracic pressure EP_{RA} increased markedly, while EP_{LA} decreased somewhat. The change of pressure at the V point was of the order of +40 mm. for EP_{RA} and -10 mm. for EP_{LA} . In this particular instance the effective right atrial pressure was increased so that it exceeded effective left atrial pressure during most of the cardiac cycle. This is the only situation thus far encountered in which EP_{RA} could be made to exceed EP_{LA} . It is unfortunate that more of these experiments could not be obtained because experiments such as these represent the closest ap-

proach that can be made in the anesthetized dog to simulating the act of straining in man.

Inter-atrial Pressure Gradients. The inter-atrial pressure gradient may be calculated by subtracting EP_{RA} from EP_{LA} at simultaneously occurring points and the differential pressure thus obtained plotted as shown on figure 3. A gradient whose direction is from left atrium to right atrium is plotted as a positive pressure and a gradient from right to left as a negative one. The magnitude of the gradient at any point is expressed in millimeters of saline.

In figure 3, *plots A, B, and C* represent the fluctuations of the inter-atrial pressure gradient during spontaneous respiration, Müller's experiment, and mild positive pressure lung inflation, respectively. The patterns of gradient fluctuation which occur during these types of respiratory variations are identical. As can be seen from the plots, the inter-atrial pressure gradient becomes less positive when intrathoracic pressure decreases and more positive when intrathoracic pressure increases. In other words, a decrease of intrathoracic pressure favors the development of a right to left inter-atrial pressure gradient and vice versa. However, it must be kept in mind that EP_{LA} is usually much greater than EP_{RA} and, therefore, a decrease of intrathoracic pressure per se does not reverse the direction of the gradient. As shown in the plots, there is usually a brief period in each cardiac cycle during which the gradient is negative even during the period of apnea. Decreasing the intrathoracic pressure increases the magnitude of this negative gradient but does not increase the time over which it acts.

The effect of a dyspneic type of respiration (sciatic stimulation) on the inter-atrial pressure gradient is illustrated in figure 3, *plot E*. Despite the great amplitude of the intrathoracic pressure change (+178 to -97 mm. saline), the pressure gradient is affected surprisingly little. At the peak of the forced expiratory movement the gradient tends to be more positive, while at the trough of the respiratory curve it is less positive than a comparable point during normal inspiration. Nevertheless, the gradient never crosses over to a negative value.

The situation appears to be different where an active expiratory movement is maintained for a relatively long time, however. In figure 3, *plot D* is derived from the previously discussed experiment where sciatic stimulation resulted in a prolonged active expiratory movement. The gradient, which was positive at all times during normal respiration (left hand curve), reversed so that a right to left pressure gradient with a mean value of about 20 mm. saline existed for an appreciable time (right hand curve). This plot shows that a reversal of the inter-atrial pressure gradient can occur when intrathoracic pressure is increased and maintained by action of the expiratory muscles.

DISCUSSION

Opinion is divided as to what effect inspiration (decrease of intrathoracic pressure) has on atrial and pulmonary hemodynamics. According to some investigators, inspiration increases venous return, effective right atrial pressure, right ventricular discharge, and pulmonary arterial pressure. Other workers deny all but the last mentioned effect. The increase in pulmonary arterial pressure is explained by

an increase in right ventricular stroke volume in one case, and by an increase of pulmonary resistance in the other. According to the first view, pulmonary resistance decreases during inspiration and allows the pulmonary bed to accommodate the increased right ventricular output. (For references and a fuller discussion see Wiggers, 6.)

It seems possible that two views so diametrically opposed cannot ever be compromised, and it is probable that the problem will be settled by new experiments rather than by reinterpretation of the old. In this connection it is pertinent to see what the experiments reported above have to offer in support of either view. Since these experiments were not designed with this problem in mind, the information bearing directly on the question is limited.

In all our experiments effective right atrial pressure increased as a consequence of decreasing intrathoracic pressure. This contradicts the contention that inspiration produces no change in effective right atrial pressure, but offers no evidence in itself as to whether the increase is caused by increased venous return or by increased pulmonary resistance. It is suggested that the failure by some to detect the increase of effective right atrial pressure might have been because of the use of insufficiently sensitive and inaccurate manometers, together with a failure to record intrathoracic pressure in close proximity to the right atrium.

Effective left atrial pressure has not been studied in connection with the problem to the best of our knowledge, although Hamilton, Woodbury and Vogt (7) have recorded pulmonary venous pressures. The fact that effective left atrial pressure increases suggests that inspiration partially empties the venous side of the pulmonary bed toward the left atrium. This does not agree with the idea that left ventricular systolic discharge is decreased as a result of inspiration. However, an interpretation of decreased systolic discharge based on a decrease of aortic pulse pressure might be fallacious, considering that an increase of aortic volume (as a result of decreased intrathoracic pressure) with a constant left ventricular stroke volume might also decrease pulse pressure.

These data indicate that effective right and left atrial pressure changes caused by change of intrathoracic pressure are similar as to direction, but differ as to magnitude. Both effective pressures increase as a result of decreasing intrathoracic pressure and decrease when intrathoracic pressure rises. Because of the fact that effective right atrial pressure increases more than the left when intrathoracic pressure decreases, the inter-atrial pressure gradient exhibits predictable variation with changes of intrathoracic pressure. The variation is such that the gradient becomes less positive with decrease of intrathoracic pressure and more positive when intrathoracic pressure increases. This fluctuation is slight during normal spontaneous respiration and mild positive pressure lung inflation, but more marked in the case of Müller's experiment and dyspnea.

Since decreasing intrathoracic pressure does reduce the positive inter-atrial pressure gradient, it is obvious that this effect would be of importance in the presence of complications which tend to abolish the normal pressure difference between the atria, e.g. pulmonary stenosis, cor pulmonale, right ventricular failure and, possibly, inter-atrial septal defect. A series of experiments previously reported (1) revealed

that about 10 per cent of open-chest dogs had right atrial pressures equal to or greater than the left atrial pressure. This situation was not encountered in the present series. If the situation should exist in the closed-chest dog, however, it is possible that right to left gradients would increase or be developed as a result of a decrease of intrathoracic pressure.

The reversal of the left to right gradient as a result of maintained active expiratory effort is worthy of comment. Apparently, the increase of intrathoracic pressure per se is not the sole cause of the reversal because an increase of intrathoracic pressure during dyspnea, positive pressure lung inflation or abdominal compression did not result in a reversal of the gradient, although sizable increases of intrathoracic pressure were obtained in each instance. The most apparent factor contributing to the difference in results is the fact that where the gradient was reversed there was a maintained contraction of expiratory muscles. In contrast, the gradient was unaffected by the brief contraction of expiratory muscles during dyspnea or by the maintained increase of intrathoracic pressure caused by abdominal compression in which expiratory muscles did not participate. It appears, then, that at least two factors are concerned in bringing about a reversal of the inter-atrial pressure gradient: *a*) active participation of expiratory muscles and *b*) increased intrathoracic pressure acting over sufficient time. It would seem that active contraction of expiratory muscles exerts some effect on intrathoracic blood flow apart from the effect produced by increasing intrathoracic pressure. The time factor suggests that a new equilibrium is established. The details of these mechanisms are not clear at present.

SUMMARY

Simultaneously recorded intrathoracic pressure and right and left atrial pressures were obtained in closed-chest dogs. Instantaneous effective right and left atrial pressures were calculated during normal respiration and in states where there were abnormally great fluctuations of intrathoracic pressure. During the apneic phase of normal respiration effective left atrial pressure exceeds effective right atrial pressure. A decrease of intrathoracic pressure (inspiration) during normal respiration increases effective right atrial pressure, but has no significant effect on effective left atrial pressure. Greatly decreased intrathoracic pressure (Müller's experiment) results in an increase of both right and left effective atrial pressure, but the former increases more promptly and to a greater extent than the latter.

Increased intrathoracic pressure occasioned by mild positive pressure lung inflation, gentle abdominal pressure and dyspnea from sciatic nerve stimulation generally decreased both right and left effective atrial pressures, the decrease of effective right atrial pressure predominating. In one case where sciatic stimulation produced a maintained active expiratory attempt (with trachea occluded), effective right atrial pressure exceeded effective left atrial pressure.

It is concluded that physiologic changes of intrathoracic pressure result in slight but predictable changes of effective atrial pressure. The magnitude of the left to right inter-atrial pressure gradient reflects these changes of effective right and left atrial pressures, the gradient becoming less with decreasing intrathoracic pressure and vice versa. However, a decrease of intrathoracic pressure seldom reverses the

direction of the gradient except in the presence of pathological complications, which tend to equalize right and left atrial pressures.

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A QUANTITATIVE STUDY OF THE REDUCTION OF GASTRIC ACID SECRETION ASSOCIATED WITH PYREXIA*

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THAT a rise in body temperature is frequently associated with depression of acid secretion by the stomach has been long known and has been frequently confirmed. (See reference (1) for bibliography.) However, little information is available on the quantitative relationship between the magnitude of the rise in temperature and the degree of depression of acid secretion or on the relationship of the time course of the two events.

The present experiments were performed in order to gain information on these aspects of the subject. The 'continuous histamine method' (2) which we used made it possible to measure inhibition accurately and to follow its time course. The repeated single injection method used in previous studies is not so accurate nor does it permit accurate correlation of the time course of fever and secretory inhibition.

MATERIALS AND METHODS

In 4 dogs with total gastric pouches, gastric acid secretion was stimulated by the subcutaneous injection of 0.0125 mg. of histamine dihydrochloride every 10 minutes during the test. The secretions collected during the first hour were discarded because the secretory rate had not yet stabilized. The second hour served as a control period, during which the secretory rate was relatively constant. Then one of the 2 pyrogenic substances used was injected intravenously, and collections were continued for a period of 3 or more hours. The 2 pyrogens used in this study were Pyromen¹ and pyrexin². Pyromen, a purified material obtained from cultures of *Pseudomonas aeruginosa* (3), was administered in doses ranging from 0.5 µg/kg. to 10 µg/kg. Pyrexin is prepared from the pleural cavity exudate of dogs with sterile pleuritis induced by turpentine (4). It was injected in doses ranging from 2 to 30 mg. In most tests rectal temperatures were taken every hour with a clinical thermometer.

Gastric juice was collected every 20 minutes. The volume was measured and free and total acid content were determined by titration with 0.03N NaOH using p-dimethylaminoazobenzene and phenolphthalein as indicators.

RESULTS

In table 1 are presented the results of 7 tests in which the 1.0 µg/kg. dose of Pyromen was used. Data from the other tests in this study were tabulated in a similar manner, and all calculations were made on the measurement of free HCl.

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¹ Supplied by Dr. N. M. Nasset, Baxter Laboratories, Inc., Morton Grove, Ill.

² Supplied by Dr. V. Menkin, Duke University Medical School, Durham, N. C.

TABLE 1. INHIBITION OF GASTRIC SECRETION AND RISE IN BODY TEMPERATURE FOLLOWING INTRAVENOUS INJECTION OF PYROMEN, $\mu\text{G/KG}$.

TEST	CONTROL HR. mm. HCl	FIRST HR.		SECOND HR.		THIRD HR.		MEAN AVERAGE FOR 3 HOURS		AVERAGE PER CENT INHIBITION FOR 3 HOURS
		mm. HCl	Temp. Rise °F.	mm. HCl	Temp. Rise °F.	mm. HCl	Temp. Rise °F.	mm. HCl	Temp. Rise °F.	
1	1.119	1.137	3.1	0.072	3.4	0.006	2.8	0.405	3.10	63.8
2	3.447	3.816	0.4	1.098	1.3	0.195	2.0	1.703	1.23	50.6
3	4.044	4.176	0.7	1.035	2.2	0.096	2.9	1.769	1.93	56.2
4	2.508	2.163	2.0	0.447	0.4	0.108	-0.4	0.906	0.67	63.9
5	1.656	2.190	0.7	2.205	0.4	1.200	-0.4	1.865	0.23	-12.7
6	0.234	0.282	0.4	0.144	0.8	0	0.1	0.132	0.43	43.6
7	1.941	2.475	0.1	1.569	0.7	2.145	0.4	2.063	0.40	-6.2
Average....	2.136	2.320	1.06	0.934	1.33	0.536	1.06	1.263	1.14	37.0

The average control secretory rate was 2.136 mm/hr. \pm 0.498.

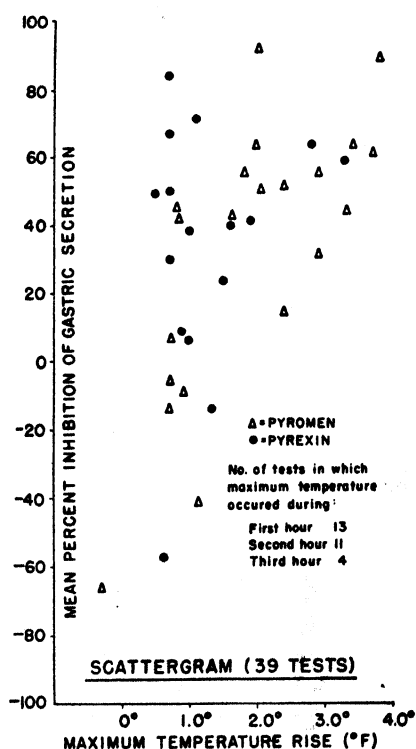


Fig. 1. RESULTS of each of 39 tests in terms of maximum temperature rise and mean per cent inhibition of gastric secretion.

Figure 1 is a scattergram showing the results of each of 39 tests in terms of maximum temperature rise and mean per cent inhibition of gastric secretion. The points are too widely distributed to describe any more than a general trend, which is that the degree of inhibition increases as the temperature rises. Also equally scattered are those points representing only the tests in which the maximum temperature rise occurred during the first, second, or the third hour. However, when the results are plotted as averages, significant correlations become evident.

The results of 21 of the tests using bacterial pyrogen are presented in figure 2. Here, all 3 combinations of the independent variable (dose), and the 2 dependent variables (temperature rise and inhibition of secretion) are plotted on the same graph. The highest dose used ($10 \mu\text{g/kg.}$), produced an average temperature rise of 3.0°F. , and an average of 72 per cent inhibition of secretion for the 3-hour period.

In figure 3 are presented the results of 18 tests in which pyrexin was injected.

These results describe a pattern similar to that produced by Pyromen. In both instances the relation between log dose of pyrogen and pyrexia or inhibition is linear over a large portion of the range of doses studied.

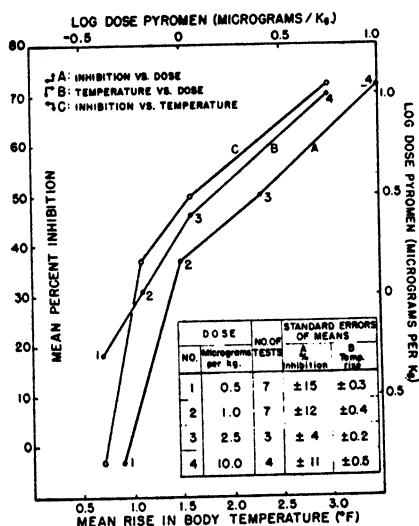


Fig. 2. (Left) EFFECTS on body temperature and gastric secretion produced by bacterial pyrogen.

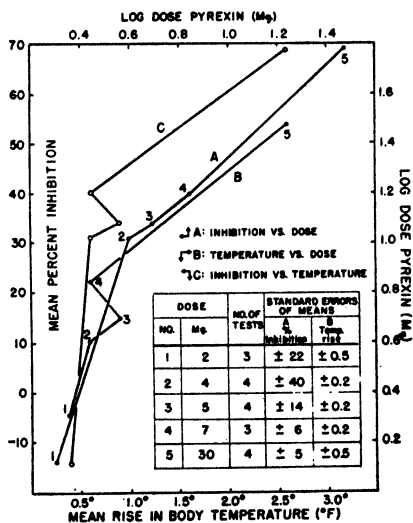
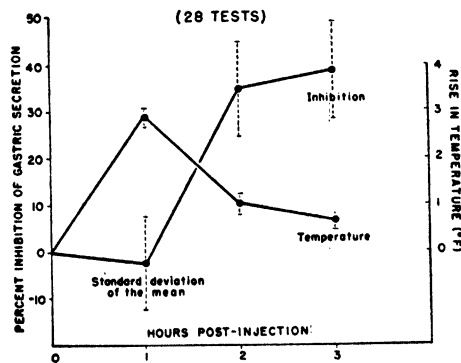


Fig. 3. (Right) EFFECT on body temperature and gastric secretion produced by pyrexin.

Fig. 4. COURSE of temperature rise and gastric secretory inhibition for 3 hours following injection of pyrogen.



In no test, using either Pyromen or pyrexin, was there depression of secretion without a rise in body temperature. In 5 tests the temperature had returned to normal 3 hours after the injection, while at the same time there was definite inhibition of secretion.

The course of pyrexia and gastric secretory inhibition is shown in figure 4, representing the average of 28 tests in which temperature was recorded for each of the 3 hours, regardless of the dose of pyrogen. The pattern shown here suggests that

the inhibitory response lags behind the temperature response for a period of at least one hour.

This lag also shows up when the results of individual tests are inspected (fig. 5). In each of the 3 tests shown here the onset of fever occurs during the first hour, while at the same time the secretory rate is not significantly changed. In test *H* the body temperature had returned to normal 5 hours after the injection, but gastric secretion was still inhibited 37 per cent.

DISCUSSION

It should be emphasized that in no test did we observe reduction in gastric acidity without elevation in body temperature. In fact, even those doses of pyrogenic materials which produced a slight rise in body temperature (up to about $0.5^{\circ}\text{F}.$) did not cause inhibition of secretion. This is contradictory to the statement of Necheles *et al.* (5) who showed that subpyrogenic doses of pyrogenic materials inhibit gastric motility, and stated that they also inhibited gastric secretion, although no data on gastric secretion were presented.

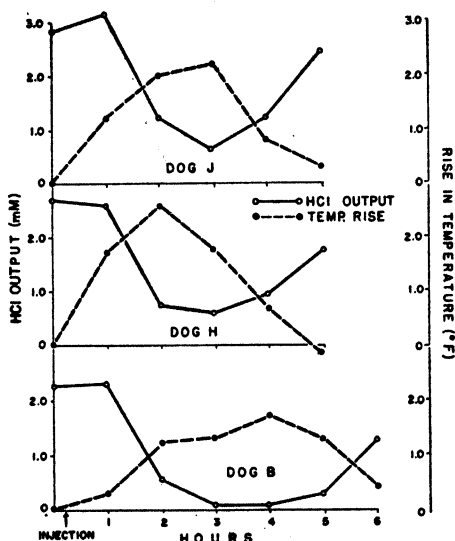


Fig. 5. THREE TESTS showing temperature rise and gastric inhibition following injection of pyrogen ($\mu\text{g}/\text{kg}.$)

The mechanism of the inhibition of gastric secretion which is associated with pyrexia is not known. A full discussion of the theoretical possibilities will not be undertaken here. Only two points will be mentioned because the findings of the present study bear upon these possibilities. First, the lag in the onset and subsidence of the secretory depression in relation to the rise in body temperature speaks against the temperature

per se being the agent causing inhibition. Second, the fact that the secretory rate returns to control levels as the fever subsides, rules out the possibility that any change which would require repletion from an external source, such as dehydration, is responsible.

It is possible to prepare urogastrone (6) and enterogastrone (7) free of pyrogen, although unless special precautions are taken these extracts are frequently contaminated. Because of the important effect which contamination with pyrogen has on the interpretation of the results of experiments with enterogastrone and urogastrone and other biological extracts and drugs, the following points revealed by the present study should be stressed: *a*) During the first hour after injection of pyrogen there is usually no inhibition of secretion although the temperature rise

may be maximal at this time. Injections of enterogastrone (2) and urogastrone (8) depress secretion almost immediately, and this difference serves as a valuable guide to the investigator in determining whether depressant activity is due to these specific substances, or whether it is due to pyrogenic contaminants. *b*) Several hours after injection of pyrogen, the temperature may have returned to normal, but secretion may still be inhibited. If temperature were to be measured only at this time it would appear that inhibition of secretion without rise in temperature had occurred.

SUMMARY

Pyrexia was produced by intravenous injection of pyrexin or of bacterial pyrogen and its effect upon the rate of gastric secretion induced in gastric pouch dogs by injection of histamine every 10 minutes was observed. For both pyrogenic materials, the relationship between the logarithm of the dose of pyrogen and the degree of pyrexia was linear over most of the range studied. The relationship between the logarithm of the dose of pyrogen and the degree of gastric secretory inhibition was also linear over most of the range studied.

In no individual test did gastric secretory inhibition occur without pyrexia. There was a lag of about one hour between onset of pyrexia and the onset of inhibition; there was a similar lag between the subsidence of pyrexia and the return of secretion to control levels. In this respect the inhibition produced by pyrogen differs from that produced by urogastrone and enterogastrone concentrates. Because of these lags, early in the course of a test, pyrexia often occurred without secretory inhibition, whereas late in the test secretory inhibition without pyrexia often was seen. Pyrexia is, therefore, considered not to be directly responsible for the inhibition of secretory activity.

The technical assistance of C. E. Rosiere is gratefully acknowledged.

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NATURE OF ACTION OF THE LABILE FACTOR IN FORMATION OF THROMBIN¹

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IT IS now accepted that, in addition to thromboplastin and calcium, a third factor is required for the formation of thrombin. This factor was discovered in 1943 by Quick (1) and again independently the following year by Owren (2), both authors utilizing the one-stage method. Quick named the new agent component A, but later gave it the non-committal term, labile factor (3), because it loses activity on storage. Owren designated the agent as factor V. The fact that the prothrombin time becomes proportionately delayed as this factor is diminished suggests that the production of thrombin is reduced but the theoretical objection can be raised that should the new factor act as an accelerator, the activation of prothrombin would only be slowed. The recent procedure developed by Quick and Stefanini (4) for isolating and assaying free prothrombin (component A) by means of adsorption with tricalcium phosphate and elution with sodium citrate offers a direct approach for determining whether or not the labile factor acts stoichiometrically, or as an accelerator. The basic experiment devised was as follows: To stored oxalated plasma, a fixed excess of thromboplastin, an optimum quantity of calcium and varying amounts of labile factor were added, the fibrin was removed and the prothrombin remaining in the serum was estimated by the adsorption and elution method. By this means, the effect of the labile factor on prothrombin consumption was quantitatively determined since the other agents in the reaction were maintained constant.

EXPERIMENTAL

Depletion of the Labile Factor in Plasma by Storage. The plasma obtained from oxalated human blood (1 volume of 0.1 M sodium oxalate to 9 volumes of blood) was placed in an ordinary refrigerator. When the prothrombin time became 45 to 60 seconds, which usually required 8 to 14 days, the plasma was considered suitable for the study described in this paper.

Influence of the Labile Factor on the Consumption of Prothrombin. Two cc. of stored human plasma were mixed with 0.1 cc. thromboplastin (Quick's), 0.1 cc. of diluted deprothrombinized rabbit plasma, and 0.4 cc. of 0.1 M calcium chloride. The fibrin was removed by wrapping it on a stirring rod. The prothrombin remaining in the serum was determined by adsorption with tricalcium phosphate and elution with sodium citrate, according to the procedure of Quick and Stefanini (4), 15 minutes after the removal of fibrin. Rabbit plasma previously treated with $\text{Ca}_3(\text{PO}_4)_2$ gel 0.012 M to remove prothrombin served as the source of labile factor, since it contains 50 times as much as human plasma. By means of serial dilutions of phosphate rabbit plasma the amount of labile factor in the reaction mixture was quantitatively varied.

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DISCUSSION

The results summarized in table 1 clearly show that the consumption of prothrombin is very incomplete when the concentration of the labile factor is low even though thromboplastin is in excess and the concentration of calcium is optimal. The poor activation of prothrombin when there is a lack of labile factor was already observed by Quick in 1943 (1) but it was not possible at that time to state with certainty whether or not the new factor functioned stoichiometrically or as an accelerator. Recently, Quick and Stefanini (4) showed that the prothrombin time of fresh human plasma was not shortened by the addition of rabbit plasma treated with tricalcium phosphate although the labile factor was thereby increased ten-fold or more. These findings clearly suggest that the labile factor does not act as an accelerator. The results reported in this paper confirm this conclusion decisively. It appears very likely that in the complete absence of the labile factor no thrombin is formed. Owren (5) has reached a similar conclusion.

TABLE 1. CONSUMPTION OF PROTHROMBIN WHEN STORED HUMAN PLASMA COAGULATES IN THE PRESENCE OF EXCESS THROMBOPLASTIN, AN OPTIMUM AMOUNT OF CALCIUM AND VARYING QUANTITIES OF LABILE FACTOR

VOLUME OF UNDILUTED $\text{Ca}_3(\text{PO}_4)_2$ RABBIT PLASMA ADDED TO MIXTURE ¹	PROTHROMBIN TIME ² RABBIT $\text{Ca}_3(\text{PO}_4)_2$ PLASMA: 0.09 cc. ELUATE FROM SERUM: 0.01 cc.	REMARKS
cc.	sec.	
0.0	9	Before coagulation
0.0	11 ³	After coagulation
0.001	14 ³	" "
0.002	18	" "
0.005	22	" "
0.050	24	" "
0.100	25	" "

¹Mixture: Oxalated human stored plasma, 2.0 cc.; thromboplastin emulsion, 0.1 cc.; calcium chloride 0.1 M, 0.4 cc.; deprothrombinized diluted rabbit plasma, 0.1 cc.

² The eluate was prepared 15 minutes after coagulation was complete. ³ No further consumption of prothrombin occurred when the incubation period was prolonged to one hour.

Recently reported experimental findings indicate that the first reaction in coagulation, the activation of thromboplastinogen to thromboplastin, is enzymatic (6, 7), but the subsequent reaction or reactions that bring about the formation of thrombin are stoichiometric. A study of the interaction of thromboplastin and prothrombin led Mertz, Seegers and Smith (8) to interpret the reaction as stoichiometric. Later Quick (9) reported findings showing that calcium likewise enters this reaction stoichiometrically. Recently Lewis and Ferguson (10) using prothrombin and Ac-globulin supplied to them by Seegers state that their data "indicates that thromboplastin, calcium, Ac-globulin and prothrombin react together in such a fashion that the quantity of thrombin formed is dependent upon the quantity of each in the original mixture."

None of the basic agents in the reaction appear to act as accelerators or catalysts and the results of various investigations suggest that the speed of the reaction follows

the law of mass action. It is unlikely that the 4 agents interact simultaneously but it is not known in what order these factors enter the reaction. For the purpose of classifying the hemorrhagic diseases, it is convenient to group prothrombin, the labile factor and calcium as the prothrombin complex since the prothrombin time is normal when the concentration of these 3 are at the physiological level.

Owren (5) and Fantl and Nance (11) conclude that their agents, factor *V* and accelerator factor respectively, are identical with the labile factor. Fahey, Ware and Seegers (12), after correcting their early conclusions concerning the stability of their Ac-globulin (13), likewise are inclined to believe that their factor, and the original component *A* (labile factor) are the same. Fantl and Nance, and Seegers and his associates consider the new agent to be an accelerator. Owren postulates that factor *V* enzymatically reacts with cytokinase to form factor *VI* or prothrombinase which with ionic calcium catalytically converts prothrombin to thrombin. The views of these 3 groups of investigators are at variance with those of the writers who hold that the labile factor does not act as an accelerator or catalyst and who have presented the evidence to show that its action is stoichiometric.

In seeking the reason why the reaction that brings about the formation of thrombin has not been generally recognized as being stoichiometric, it should be pointed out that thrombin is definitely enzymatic in its action, and recently findings have been reported that thromboplastin exists in a precursor state which is activated by a platelet enzyme. The formation of thrombin coming between 2 enzymatic reactions can, if caution is not taken, be itself looked upon as enzymatic. This probably accounts for the erroneous assumptions that thromboplastin, ionized calcium and now the labile factor are catalysts.

A more cogent reason perhaps for mistaking the action of the labile factor as catalytic can be found in the recent discovery of Quick and Stefanini (14) that prothrombin (component *A*) exists partly free and partly in a precursor state. Not only does the labile factor diminish on storing plasma in glass but the inactive prothrombin becomes changed to the active form so that the concentration of the latter is more than doubled in human plasma. Therefore, on adding labile factor to stored plasma, the prothrombin time actually becomes shorter than normal. This fact can easily be misinterpreted as an accelerating effect of the labile factor, whereas the shorter prothrombin time is caused by a true increase in free prothrombin as determined by the new adsorption and elution method. It is highly probable that the assumption, that the convertibility of prothrombin per se can be altered, may be reinterpreted as a conversion of prothrombin precursor to the active state.

In the course of this study it was also observed that even when the labile factor and thromboplastin were in excess, a small amount of prothrombin remained unconverted after coagulation both in fresh and in stored plasma. Further study will be required to explain this observation.

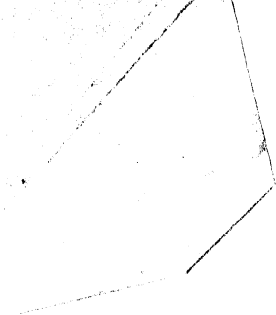
SUMMARY

When excess thromboplastin and an optimum amount of calcium are added to stored oxalated plasma, only a small quantity of prothrombin is consumed. On adding increasing quantities of labile factor in the form of diluted deprothrombinized rabbit

plasma, the consumption of prothrombin is progressively increased. This indicates that the role of the labile factor in the formation of thrombin, like that of thromboplastin and calcium, is stoichiometric.

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Index to Volume 160

- ACETYLCHOLINE** in summation of stimuli, 375
- Acoustic cortex, 395
- Actomyosin and the uterus, 46
- Adrenal cortex
- adrenergic agents, 490
 - response to stress, 499
 - and spleen in relation to leucocytes, 75
- Adrenal cortical extract (ACE)
- antagonism to desoxycorticosterone, 217
 - in shock, 83
- Adrenaline and respiration, 485
- Adrenals and extracellular sodium and chloride depletion, 89
- Adrenergic agents and adrenal cortex, 490
- Adrenocorticotrophic hormone (ACTH), antagonism to desoxycorticosterone, 217
- ALEXANDER, W. F., 441
- Alloxan
- and changes in blood sugar, 228
 - and liver gluconeogenesis, 107
- Altitude and decompression injury, 361
- Amines
- in blood during hypertension, 409
 - convulsions in cerebellum and cerebrum induced by, 195
 - and inhibition of cholinesterase, 187
- Anabolism
- and androgens, 62
 - protein and hormones, 66
- Androgens
- and protein anabolism, 62
 - protein anabolic property of, 53
- Anesthesia
- and blood sugar response to alloxan, 228
 - ether and barbiturate, and blood changes, 277
 - sodium pentobarbital and ether effects on rate of flow and cell content of thoracic duct lymph, 9
- Anoxia
- and injury, 361
 - and respiration, 138
- Anticholinesterases and summation of stimuli, 375
- Antiproteolytic activity and ulcer formation, 348
- Apneusis and respiration, 385
- Arterial pressure
- and body temperature, 402
 - and electrical alternation, 366
 - and hypotension, 519
 - and liver, 421
- Ascorbic acid in adrenal cortex and spleen, 75
- Atrial pressures, 556
- Auricular fibrillation and coronary blood flow, 177
- BAIN, J. A.**, Inhibition of rat brain cholinesterase by β -chlorinated amines, 187; 195
- BALL, S. M., 353
- BANCROFT, R. W., 103
- BANKS, E. M., 462
- BEALL, B., 62
- BERGHOUT, J., 479
- BERLINER, R. W., T. J. KENNEDY, JR. AND J. G. HILTON. Renal clearance of ferrocyanide in the dog, 325
- BERNHEIM, F., 31
- BEST, C. H., 228
- Beta cell granulation and alloxan, 228
- BEYER, K. H., H. F. RUSSO, S. R. GASS, K. M. WILHOYTE AND A. A. PITT. Renal tubular elimination of N^1 -methylnicotinamide, 311
- BIGELOW, W. G., W. K. LINDSAY, R. C. HARRISON, R. A. GORDON AND W. F. GREENWOOD. Oxygen transport and utilization in dogs at low-body temperatures, 125
- BING, R. J., 149
- BLACK, H., 163
- BLICKENSTAFF, D. AND M. I. GROSSMAN. A quantitative study of the reduction of gastric acid secretion associated with pyrexia, 567
- Blood
- amine content during hypertension, 409
 - calcium and citrate content, 335
 - citric acid content, 330, 341
 - gas tensions in, 163
 - glucose removal, 506
 - homologous, and hypertonic solutions, 509, 519
 - renal flow, 547
 - vascular reactivity, 421
 - tocopherol levels, 259
- Blood changes
- and anesthesia, 277
 - following evisceration, 247
 - and splenectomy, 295

- Blood flow**
 and cardiac oxygen, 149
 and lumbar sympathetic trunk, 441
 and sympatholytic effects of cinchona alkaloids, 212
 techniques for measuring, 183
- Blood pressure**
 and auricular fibrillation, 177
 and diet in renal hypertension, 31
 and renal clearance in hypertension, 21
- Blood sugar changes**
 following alloxan, 228
 following partial pancreatectomy, 103
- BLUMENTHAL, M. R.**, 177
- Body weight**
 effects of protein anabolism and hormones, 66
 and steroids, 62
- Brain**
 acoustic area, 395
 stimulation on phosphate esters of, 203
 turtle, 402
- BRECHER, G. A.**, 556
- BRECKENRIDGE, C. G.**, 485
- BRECKENRIDGE, C. G. AND H. E. HOFF.** Pontine and medullary regulation in the cat, 385
- BRIDGES, W. C.**, 306
- BRUCER, M., G. L. HERMAN AND H. G. SWANN.** Interrelationship of the cardio-respiratory events in anoxia, 138
- Bubble flow meter and renal blood flow**, 547
- CALCIUM**
 and citric acid, 330
 serum and thyroid function, 341
- Carbon dioxide**, effects on convulsions in cerebellum and cerebrum, 195
- Cardiac input**, measured with a rotameter, 183
- Cardiac output**
 and auricular fibrillation, 177
 and hypotension after hypertonic solutions, 15
 inhibition, 552
 measured by direct Fick method, 183
 oxygen consumption and coronary blood flow, 149
- CARRASCO-FORMIGUERA, R. AND M. T. MENDOZA.** Impairment of liver gluconeogenesis from lactic acid as result of alloxan action, 107
- Cerebral cortex**, acoustic area in, 395
- CHANG, T. S.**, 335, 341
- CHANG, T. S. AND S. FREEMAN.** Citric acid and its relation to serum and urinary calcium, 330
- CHENG, C.**, 217
- Chloride excretion**, 536
- Chloride and extracellular sodium in adrenals**, 89
- Cholinesterase inhibition by amines**, 187
- Cinchona alkaloids and renal blood flow and blood pressure**, 212
- Citric acid**
 and hypercalcemia, 335
 and thyroparathyroidectomy, 341
 and urinary calcium, 330
- CIZEK, L. J. AND J. H. HOLMES.** Chloride excretion during osmotic diuresis in the dog, 536
- CLIFFTON, E. E. AND L. E. YOUNG.** Antiproteolytic activity of serum from dogs with experimentally-induced peptic ulcers, 348
- COHN, R. AND H. PARSONS.** Relationship of portal hypertension and irreversibility of shock, 437
- COLLINS-WILLIAMS, J.**, 228
- Conditioned reflexes in young puppies**, 462
- CONN, H. L., JR. AND K. MARKLEY.** Simultaneous comparison of renal blood flow as measured by the Fick principle and by the bubble flow meter, 547
- Convulsions in cerebellum and cerebrum induced by amines**, 195
- COREY, E. L.**, 361
- Coronary blood flow**
 and auricular fibrillation, 177
 and oxygen consumption, 149
- Coronary occlusion**
 and electrical alternation, 366
- COX, J. W.**, 441
- CSAPÓ, Á.** Actomyosin of the uterus, 46
- CUNNINGHAM, J. E.**, 485
- DAVIS, A. K.**, 89
- DAWSON, R. M. C. AND D. RICHTER.** Effect of stimulation on the phosphate esters of the brain, 203
- Decompression injury and anoxia**, 361
- Depressor substance and hypotension**, 509
- Desoxycorticosterone (DCA)**
 antagonism of adrenocorticotrophic hormone and adrenal cortical extract, 217
 in shock, 83
- Dextrose and diabetes insipidus**, 321
- DEYRUP, I. J.**, 15
- DEYRUP, I. J. AND W. W. WALCOTT.** Observations on hypotension following intravenous injection of strongly hypertonic solutions mixed with homologous blood, 519
- Studies on mechanism of potentiation of circulatory effects of hypertonic solu-**

- tions resulting from admixture of these solutions with homologous blood, 509
- Diabetes**
production by partial pancreatectomy, 103
and renal function, 321
- Diet**
and blood sugar response to alloxan, 228
and renal hypertension, 31
- Diuresis**, osmotic, 536
- DJU, M. Y., M. L. QUAIFE AND P. L. HARRIS.**
Utilization of pure α -, γ -, and δ -tocopherols by laying hens, 259
- DREYFUSS, F.**, 177
- Drugs**
adrenaline apnea, 485
and blood changes, 277
and epinephrine toxicity, 479
as inhibitors of cholinesterase, 187
pilocarpine, 467
polarization of spinal cord elements, 451
and renal blood flow and blood pressure, 212
sodium pentobarbital and epinephrine release, 499
and vascular reactivity, 421
- Duct ligation and pancreatic function**, 115
- DURY, A.**, Leucocyte picture of the rat: relation of adrenal and spleen, 75
- EASLER, C. A.**, 462
- EICHELBERGER, L., AND M. ROMA.** Water and electrolyte distribution in blood and tissues in splenectomized dogs before and after hypotonic saline injections, 295
- Electrical activity of acoustic cortex**, 395
- Electrical alternation and coronary occlusion**, 366
- Electrolytes**
absorption of, 264
and electroshock threshold, 217
and sea water, 291
and splenectomy, 295
- Epinephrine**
and adrenal cortical activity, 490
and anesthesia, 499
toxicity, 479
- Eserine and pupillary response**, 474
- Esters**, brain phosphate, 203
- Estrogen degradation by liver**, 41
- Evisceration, blood changes following**, 247
- Extracellular fluid, measurement**, 526
- FEIGEN, G. A.**, 451
- FERGUSON, D.**, 402
- Ferritin and hemoglobin iron**, 1
- Ferrocyanide, renal clearance of**, 325
- FIELD, L.**, 306
- FIELD, N. S.**, 41
- Fick method for measuring cardiac output**, 183
- Fick principle and renal blood flow**, 547
- FLANAGAN, J. B., A. K. DAVIS AND R. R. OVERMAN.** Mechanism of extracellular sodium and chloride depletion in the adrenalectomized dog, 89
- FRANK, C. W.**, 177
- FREEMAN, S.**, 330
- FREEMAN, S. AND T. S. CHANG.**
Effect of thyroparathyroidectomy and vitamin D upon serum calcium and citric acid of normal and nephrectomized dogs, 341
Role of the kidney and of citric acid in production of a transient hypercalcemia following nephrectomy, 335
- FREINKEL, N.**, 532
- FREY, J. S.**, 375
- FULLER, J. L., C. A. EASLER AND E. M. BANKS.**
Formation of conditioned avoidance responses in young puppies, 462
- GANOID**, reflex cardiac inhibition in, 552
- Gas tensions in blood**, 163
- GASS, S. R.**, 311
- Gastric acid secretion**, 567
- GESELL, R. AND J. S. FREY.** Temporal summation of stimuli studied with the aid of anticholinesterases, 375
- Glomerular filtration**
and diabetes insipidus, 321
ferrocyanide excretion, 325
- Glomerular filtration rate and renal sodium excretion**, 306
- Glucose**
and brain metabolism, 203
excretion following partial pancreatectomy, 103
and survival time following evisceration, 122
tolerance of rat for, 506
- GOODMAN, L. S.**, 217
- GORDON, R. A.**, 125
- GRANT, R.** Emotional hypothermia in rabbits, 285
- GRAY, F.**, 306
- GREEN, D. W., W. C. BRIDGES, A. D. JOHNSON, J. H. LEHMAN, F. GRAY AND L. FIELD.** Relation of glomerular filtration rate and sodium tubular rejection fraction to renal sodium excretion, 306

GREENWOOD, W. F., 125

GROSSMAN, M. I., 115, 567

HALL, W. M. AND E. L. COREY. Anoxia in explosive decompression injury, 361

HAMMERSTROM, R. N., 479

HAMPTON, J. K., JR. AND H. S. MAYERSON. Hemoglobin iron as a stimulus for production of ferritin by kidney, 1

HANDLEY, C. A. AND A. D. KELLER. Changes in renal functions associated with diabetes insipidus precipitated by anterior hypothalamic lesions, 321

HANDLER, P. AND F. BERNHEIM. Importance of dietary protein, calories and salt in experimental renal hypertension, 31

HARRIS, P. L., 259

HARRISON, R. C., 125

Heart

inhibition, 552

ate and electrical alternation, 366

HELLERSTEIN, H. K. AND I. M. LIEBOW. Electrical alternation in experimental coronary artery occlusion, 366

Hemodynamics, right and left atrial pressures, 556

Hemoglobin iron as stimulus for production of ferritin by kidney, 1

HENDERSON, W. P., 441

HERMAN, G. L., 138

HERTZMAN, A. B., 441

HIATT, E. P. Sympatholytic effects of quinine and quinidine, 212

HILTON, J. G., 325

Histamine

and gastric acid secretion, 567

and ulcer formation, 348

HOFF, H. E., 385

HOFF, H. E., C. G. BRECKENRIDGE AND J. E. CUNNINGHAM. Adrenaline apnea in the medullary animal, 485

HOLMES, J. H., 536

HUGGINS, R. A., E. L. SMITH AND M. A. SINCLAIR. Comparison of the cardiac input measured with a rotameter and output determined by the direct Fick method in open-chest dogs, 183

HUNGERFORD, G. F. AND W. O. REINHARDT. Comparison of effects of sodium pentobarbital or ether-induced anesthesia on rate of flow and cell content of rat thoracic duct lymph, 9

Hypercalcemia, kidney and citric acid, 335

Hyperglycemia and glucose tolerance, 506

Hypertension

effects on blood pressure and renal clearance, 21

and pressor substances, 409

and shock, 437

Hypertonic solutions

diuresis and chloride excretion, 536

and homologous blood, 509

and hypotension, 15, 519

Hypoglycemia and alloxan, 107, 228

Hypophysis and diabetes insipidus, 321

Hypotension and hypertonic solutions, 15, 509, 519

Hypothermia

emotional, 285

and oxygen consumption, 125

Hypotonic saline injections

and splenectomy, 295

INGLE, D. J. AND J. E. NEZAMIS. Effect of temperature upon survival of eviscerate rat, 122

INGLE, D. J., J. E. NEZAMIS AND E. H. MORLEY. Effect of work upon tolerance of the normal rat for intravenously administered glucose, 506

INGLE, D. J., M. C. PRESTRUD AND J. E. NEZAMIS. Changes in the blood of the rat following evisceration, 247

Insulin

and blood sugar response to alloxan, 228

and survival time following evisceration, 122

Intrathoracic pressure, 556

Inulin and plasma-interstitial fluid, 532

Ion exchange resins and mineral metabolism, 264

Ions, sodium sulfate and renal excretion, 353

JOHNSON, A. D., 306

KANGAROO rats and utilization of sea water, 291

KATZ, L. N., 21

KEATING, R. P., 177

KELLER, A. D., 321

KENNEDY, T. J., JR., 325

Kidney

excretory components, 311

and hypercalcemia, 335

measurement of renal blood flow, 547

and sodium sulfate, 353

KISCH, B. Reflex cardiac inhibition in the ganoid *Acipenser sturio*, 552

KOCHAKIAN, C. D.

Comparison of protein anabolic properties of testosterone propionate and growth hormone in the rat, 66

- Comparison of protein anabolic property of various androgens in the castrated rat, 53
- KOCHAKIAN, C. D. AND B. BEALL. Comparison of protein anabolic property of testosterone propionate in the male and female rat, 62
- KOHN, H. I. Changes in blood of the rat during ether and barbiturate anesthesia, 277
- L**ABILE factor and thrombin, 572
- Lactic acid and alloxan action, 107
- LEHMAN, J. H., 306
- Leucocytes, relation of adrenal and spleen, 75
- LIEBOW, I. M., 366
- LINDSAY, W. K., 125
- Liver
- and arterial pressure, 421
 - and estrogen degradation, 41
 - glycogen and alloxan action, 107
- Lymph flow and anesthesia, 9
- M**CAULIFF, J. P., 264
- MCCHESNEY, E. W. AND J. P. MCAULIFF. Effects of some ion exchange resins on the mineral metabolism of rats, 264
- MAQSOOD, M. AND E. P. REINEKE. Influence of variation in environmental temperature and thyroid status on growth and feed consumption of the male mouse, 253
- MARKLEY, K., 547
- MAYERSON, H. S., 1
- Medullary regulation and respiration, 385
- Medullary respiratory center and adrenalinic apnea, 485
- MENDOZA, M. T., 107
- MERRILL, D. L., 149
- Metabolism
- and ion exchange resins, 264
 - of nicotinic acid, 311
 - thyroid status and temperature, 253
- Miosis and eserine, 474
- MORLEY, E. H., 506
- Muscle dystrophy and tocopherols, 259
- Myocardial failure and epinephrine, 479
- N**EIDLE, E. A.
- Pilocarpine sensitization in the parasympathetically denervated pupil of the cat, 467
 - Response of the parasympathetically denervated cat's pupil to eserine, 474
- Nerve impulses and acetylcholine, 375
- NEZAMIS, J. E., 122, 247, 506
- NICKERSON, M., J. BERGHOUT AND R. N. HAMMERSTROM. Mechanism of the acute lethal effect of epinephrine in rats, 479
- Nicotinamide and renal tubular elimination, 311
- Nitrogen retention
- effects of protein anabolism and hormones, 66
 - and steroids, 53, 62
- O**PDYKE, D. F. AND G. A. BRECHER. Effect of normal and abnormal changes of intrathoracic pressure on effective right and left atrial pressures, 556
- OVERMAN, R. R., 89
- Oxygen consumption and coronary blood flow, 149
- Oxygen transport and utilization at low body temperatures, 125
- P**AGE, I. H. Influence of the liver on vascular reactivity, 421
- Pancreas
- function after duct ligation, 115
 - removal in production of diabetes, 103
- Pancreozymin, ligation of pancreatic duct, 115
- PARSONS, H., 437
- PAULS, F. AND R. W. BANCROFT. Production of diabetes in the mouse by partial pancreatectomy, 103
- Peripheral resistance and arterial hypotension, 15
- Phosphocreatine levels of the brain, 203
- Pilocarpine response in parasympathetic denervation, 467
- PITT, A. A., 311
- Pituitary, anterior, 490
- Plasma
- changes following evisceration, 247
 - formation of thrombin, 572
 - and inulin, 532
- Plasma concentration in measurement of extracellular fluid, 526
- Plasma levels and anesthesia, 277
- Polarization of spinal cord elements, 451
- POLLOCK, G. H. AND J. A. BAIN. Convulsions in cerebellum and cerebrum induced by β chlorinated amines, 195
- Pontine regulation and respiration, 385
- POWERS, S. R., 149
- Pressor substances and hypertension, 409
- Pressures, intrathoracic and atrial, 556
- PRESTRUD, M. C., 247
- Proteins
- anabolic properties of steroids, 53
 - contractile, in human muscle, 46
- Pupil, parasympathetically denervated, 467, 474

- Pyrexia and gastric acid secretion, 567
 Pyrogens and emotional hypothermia, 285
- QUAIFE, M. L.**, 259
- QUICK, A. J. AND M. STEFANINI.** Nature of action of the labile factor in formation of thrombin, 572
- Quinine and quinidine, sympatholytic effects of, 212
- RANDALL, W. C., W. F. ALEXANDER, A. B. HERTZMAN, J. W. COX AND W. P. HENDERSON.** Functional significance of residual sympathetic pathways following verified lumbar sympathectomy, 441
- Reflexes, cardiac inhibition, 552
- REICHLIN, S., 490
- REINEKE, E. P., 253
- REINHARDT, W. O., 9
- Renal blood flow, measurement, 547
- Renal clearance
 of ferrocyanide, 325
 and hypertension, 21
 and nicotinamide, 311
- Renal function
 and diabetes insipidus, 321
 and ferritin, 1
 and sodium output, 306
 and sodium sulfate, 353
- Resins and mineral metabolism, 264
- Respiration
 and adrenaline, 485
 and anoxia, 138
 as factor in epinephrine toxicity, 479
 and gas tensions in blood, 163
 and medullary regulation, 385
- RICHTER, D., 203
- RODBARD, S., 21
- RODBARD, S., F. SAMSON AND D. FERGUSON. Thermosensitivity of the turtle brain as manifested by blood pressure changes, 402
- ROMA, M., 295
- RONZONI, E. Sodium pentobarbital anesthesia and the response of the adrenal cortex to stress, 499
- RONZONI, E. AND S. REICHLIN. Adrenergic agents and the adrenocortical activity of the anterior pituitary, 490
- ROOS, A. AND H. BLACK. Direct determination of partial and total tensions of respiratory gases in blood, 163
- Rotameter for measuring cardiac input, 183
- RUSSEO, H. F., 311
- SALT**, sea water utilization by kangaroo rats, 291
- SAMSON, F., 402
- SAYERS, G., 217
- SCHACHTER, D., N. FREINKEL AND I. L. SCHWARTZ. Movement of inulin between plasma and interstitial fluid, 532
- SCHMIDT-NIELSEN, B. AND K. SCHMIDT-NIELSEN. Do kangaroo rats thrive when drinking sea water?, 291
- SCHMIDT-NIELSEN, K., 291
- SCHROEDER, H. A., 409
- SCHWARTZ, I. L. Measurement of extracellular fluid by means of a constant infusion technique without collection of urine, 526; 532
- Secretin, effect of ligation of pancreatic duct on action of, 115
- SELLERS, E. A., 83
- Serum, antiproteolytic activity, 348
- Shock
 and conditioned response, 462
 and desoxycorticosterone acetate and adrenal cortical extracts, 83
 and hypertension, 437
- SINCLAIR, M. A., 183
- SMITH, E. L., 183
- Sodium and chloride in adrenals, 89
- Sodium output and renal excretion, 306
- Sodium sulfate and renal excretion, 353
- Sodium retention, influence of steroids and ACTH, 217
- SPENCER, F. C., D. L. MERRILL, S. R. POWERS AND R. J. BING. Coronary blood flow and cardiac oxygen consumption in unanesthetized dogs, 149
- Spinal cord, polarization, 451
- Spinal nerve roots and removal of lumbar sympathetic trunk, 441
- Spleen
 and adrenal cortex in relation to leucocytes, 75
 and electrolyte distribution, 295
- STAMLER, J., S. RODBARD AND L. N. KATZ. Blood pressure and renal clearances in hypertensive dogs following tissue injury, 21
- STEFANINI, M., 572
- Steroids
 anabolic properties, 53
 and electrolyte balance, 217
- STOCK, C. C. AND H. A. SCHROEDER. Pressor substances in arterial hypertension: activity and amine content of crude extracts of blood, 409

Stomach, gastric acid secretion, 567

SWANN, H. G., 138

Sympathectomy, lumbar, 441

TEMPERATURE

and emotional hypothermia, 285

and eviscerate survival, 122

and thyroid status, 253

Temperature, body

and arterial pressure, 402

following removal of lumbar sympathetic trunk, 441

and gastric acid secretion, 567

and oxygen utilization, 125

response to changes under anesthetization, 499

Temporal summation of stimuli and anticholinesterases, 375

Thyroid

and calcium and citric acid, 341

influence of temperature, 253

Thrombin, labile factor in, 572

Tocopherols, α -, γ -, δ -, utilization of, 259

Tubular rejection fraction and sodium excretion, 306

Tubular secretion and N^1 -nicotinamide, 311

TUNTURI, A. R. Physiological determination of the boundary of the acoustic area in the cerebral cortex of the dog, 395

ULCERS, antiproteolytic activity of peptic, 348

Urinary excretion and citric acid, 330, 526

Uterus, actomyosin of, 46

VAN HARREVELD, A. AND G. A. FEIGEN. Effect of polarization state of spinal cord elements, 451

Vitamin D and serum calcium and citric acid, 341

Vitamin E and tocopherols, 259

WALCOTT, W. W., 509, 519

WALCOTT, W. W. AND I. J. DEYRUP. Mechanism of hypotension following intravenous injections of strongly hypertonic solutions, 15

WANG, C. C., K. J. WANG AND M. I. GROSSMAN. Effects of ligation of pancreatic duct upon the action of secretin and pancreozymin in rabbits with a correlated histological study, 115

WANG, K. J., 115

WARD, H. P., 177

WÉGRIA, R., R. P. KEATING, H. P. WARD, F. DREYFUSS, C. W. FRANK AND M. R. BLUMEN-THAL. Effect of auricular fibrillation on coronary blood flow, 177

Weight, body, and thyroid status, 253

WERTHESEN, N. T. AND N. S. FIELD. Failure of portal system to inactivate completely ovarian secretions in immature rats, 41

WILHOYTE, K. M., 311

WOLF, A. V. AND S. M. BALL. Effect of intravenous sodium sulfate on renal excretion in the dog, 353

WOODBURY, D. M., C. CHENG, G. SAYERS AND L. S. GOODMAN. Antagonism of adreno-corticotrophic hormone and adrenal cortical extract to desoxycorticosterone: electrolytes and electroshock threshold, 217

WRENSHALL, A. G., J. COLLINS-WILLIAMS AND C. H. BEST. Initial changes in the blood sugar of the fasted anesthetized dog after alloxan, 228

YOU, S. S. AND E. A. SELLERS. Effect of desoxycorticosterone acetate and adrenal cortical extracts on survival of adrenalectomized and intact rats after burning, 83

YOUNG, L. E., 348

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